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(71) Applicants: **WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH** [US/US]; Nine Cambridge Center, Cambridge, MA 02142 (US). **THE GENERAL HOSPITAL CORPORATION** [US/US]; 55 Fruit Street, Boston, MA 02114 (US).

(72) Inventors: **CHI, Nai-Wei**; 12758 Torrey Bluff Drive, Apartment 134, San Diego, CA 92130 (US). **LODISH, Harvey, F.**; 195 Fischer Avenue, Brookline, MA 02146 (US).

(74) Agent: **COLLINS, Anne, J.**; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

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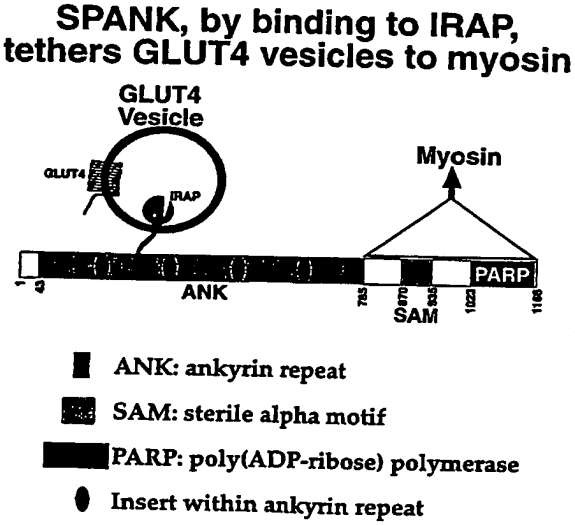
(54) Title: A NOVEL INSULIN SIGNALING MOLECULE



(57) Abstract: A novel insulin signaling protein, referred to as SPANK, DNA encoding SPANK and uses therefor.

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A NOVEL INSULIN SIGNALING MOLECULE

BACKGROUND OF THE INVENTION

Insulin enhances glucose clearance by stimulating the translocation of GLUT4 vesicles, which are endocytic vesicles containing the glucose transporter GLUT4, to the cell surface (Czech *et al.* (1999) J. Biol. Chem. 274:1865-8; Pessin et al (1999) J. Biol. Chem. 274:2593-6). In the basal state, GLUT4 vesicles are sequestered intracellularly, apparently through binding to a putative GLUT4 binding protein, referred to as G4BP. Insulin signaling relieves this sequestration and allows exocytosis of GLUT4 vesicles. A better understanding of the exocytosis of GLUT4 vesicles would provide a better understanding of the insulin-signaling pathway. A better understanding of the insulin-signaling pathway should lead to better treatment of diseases linked to aberrant insulin-signaling.

SUMMARY OF THE INVENTION

The present invention relates to a protein, referred to herein as SPANK, which co-localizes with GLUT4 in the perinuclear region of adipocytes. SPANK associates with insulin-responsive aminopeptidase (IRAP)(Keller SR et al J Biol Chem (1995) 270:23612), a major constituent of GLUT4 vesicles that is sequestered in cells in the basal state and undergoes translocation in response to insulin (in cells in an insulin-stimulated state). IRAP also co-localizes and co-purifies with GLUT4. IRAP apparently competes with GLUT4 for binding to G4BP, as evidenced by the fact that overexpression of the cytosolic domain of IRAP can cause GLUT4 translocation (Waters et al (1997) J. Biol. Chem. 272:23323-7). As described herein, SPANK has been shown to specifically interact with IRAP and, thus, is an insulin-signaling component that physically interacts with constituents of GLUT4 vesicles. SPANK has been shown to undergo stoichiometrical phosphorylation within minutes after cells are stimulated with insulin.

SPANK is a cytosolic protein that can poly(ADP-ribosyl)ate itself and other substrates *in vitro*. SPANK is comprised of three domains or regions: a SAM (sterile alpha motif) domain (Schultz et al (1997) Protein Sci :249-253), a PARP (poly (ADP-ribose) polymerase) catalytic domain (D'Amours et al (1999) Biochem J 342:249-268), and an ANK domain comprised of ankyrin repeats (Sedgewick et al (1999) Trends Biochem Sci 24:311-6); 2 linkers: one between the ANK and SAM domains, and one between the SAM and PARP domains; and an amino terminal and a short C-terminal sequence.

Also the subject of this invention are components or fragments of SPANK that bind IRAP (*e.g.*, a sufficient portion of the ankyrin repeat domain to bind the cytosolic domain of IRAP or a six amino acid region within the IRAP cytosolic domain); isolated DNA (*e.g.*, cDNA, genomic DNA) and RNA (*e.g.*, mRNA), as well as synthetic versions and variants thereof, which encode SPANK; and antibodies (both polyclonal and monoclonal) that bind SPANK. Further subjects of this invention are drugs (compounds, molecules) which are useful as therapeutics (*e.g.*, to treat insulin resistance/diabetes); methods of treatment, in which SPANK is the target, which result in enhanced GLUT4 translocation and, concomitantly, increased glucose uptake; methods of diagnosing insulin resistance/diabetes and methods of identifying or screening for drugs useful in treatment or therapy of insulin resistance and/or diabetes. A particular advantage of SPANK is that it is in contact with GLUT4 vesicles using IRAP as an adapter, is likely to be a distal effector of insulin signaling, and, thus, is a specific target for drugs that alter GLUT4 translocation/glucose uptake and are useful to treat or prevent insulin resistance or diabetes.

In one aspect, the invention features a purified, isolated, or recombinant SPANK polypeptide that has an effect on cells, wherein said effect is selected from the group consisting of modulation of GLUT4 translocation, modulation of IRAP-binding, and modulation of myosin-binding. Alternatively, the invention features a purified, isolated, or recombinant SPANK polypeptide that comprises at least 180 contiguous amino acids of the amino acid sequence set forth in the group selected from SEQ ID NO: 1, SEQ ID NO: 4, and SEQ ID NO: 6. Alternatively, the

invention features a purified, isolated, or recombinant SPANK polypeptide that is at least 82% identical to the SPANK polypeptide sequences selected from the group consisting SEQ ID NO: 1; SEQ ID NO: 4; and SEQ ID NO:6.

In a another aspect, the invention features a purified, isolated, or recombinant polynucleotide encoding said SPANK polypeptide described in the first aspect. Or, alternatively, an isolated, purified, or recombinant polynucleotide comprising a contiguous span of at least 540 nucleotides of the nucleotide sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO:3, and SEQ ID NO:5 and the complements thereof.

In a further aspect, the invention features a recombinant vector comprising said polynucleotide of claim described in the second aspect.

In yet another aspect, the invention features a recombinant cell comprising said recombinant vector of claim described in the third aspect.

In one aspect, the invention features a pharmaceutical composition comprising said SPANK polypeptide described in the first aspect and a pharmaceutically acceptable diluent.

In another aspect, the invention features a method of reducing body mass comprising providing to an individual in need of reducing body mass said pharmaceutical composition of the fifth aspect. Alternatively, the invention features a method of reducing glucose intolerance or insulin resistance comprising providing to an individual in need of reducing body mass said pharmaceutical composition of the fifth aspect. Alternatively, the invention features a method of preventing or treating an obesity-related disease or disorder comprising providing to an individual in need of such treatment said pharmaceutical composition of the fifth aspect. In preferred embodiments, said obesity-related disease or disorder is selected from the group consisting of obesity, cardiac insufficiency, coronary insufficiency, stroke, hypertension, atheromatous disease, atherosclerosis, high blood pressure, non-insulin-dependent diabetes, hyperlipidemia, hyperuricemia, and Syndrome X. Alternatively, the invention features a method of preventing or treating a muscle-related disease or disorder comprising providing to an individual in need of such treatment said pharmaceutical composition of the fifth aspect. In preferred embodiments, said muscle-related disease or disorder is selected from the group consisting of muscular dystrophy, muscle atrophy, and muscle fatigue. Preferably, the treatment comprises a pharmaceutical composition comprising a compound identified in the seventh aspect below.

In a further aspect, the invention features a method for identifying one or more compounds that modulates SPANK activity comprising:

a) contacting a cell with a candidate compound;

b) detecting a result selected from the group consisting of a modulation in GLUT4 translocation, a modulation in IRAP-binding, a modulation in myosin binding, a modulation in PARP activity, and a modulation in SPANK phosphorylation; and

c) wherein said result identifies said one or more compounds if said result differs from said result in the absence of said one or more compounds.

In preferred embodiments, the cell is also contacted with a SPANK polypeptide of the first aspect and the result identifies one or more compounds if the results differ in the presence of the compound from the absence of the compound. Preferably, said SPANK is human. Preferably said one or more compounds is selected from the group selected from SPANK polypeptide fragments and IRAP fragments. Preferably the IRAP fragments are the six nucleotides identified herein, or alternatively 4 of the six nucleotides described herein. Alternatively, said one or more compounds is selected from the group consisting of peptides, peptide libraries, non-peptide libraries, peptoids, fatty acids, lipoproteins, medicaments, antibodies, mimetics and small molecules. Mimetics are preferably synthetic mimetics of the IRAP 4 or 6 amino acid peptides described herein. Compounds are preferably small molecule inhibitors. By small molecule inhibitors includes molecules with molecular weights of 500 to 100, less, than 500, less than 100. To be included these molecules must also have activity at a level potentially useful *in vivo* such that the

dose can be tolerated by an individual and would be useful to an individual. Preferably the activity is such they require less than 100 g, less than 10 g, less than 1 g, less than 100 ng, less than 10 ng, less than 1 ng in the assays described herein or comparable assays.

In an another aspect, the invention features a non-human host animal or mammal comprising the recombinant vector described in the third aspect or recombinant cell described in the fourth aspect.

In a further aspect, the invention features a mammalian host cell comprising a recombinantly disrupted SPANK gene.

In yet another aspect, the invention features a non-human host mammal comprising a recombinantly disrupted SPANK gene.

In one aspect, the invention features an isolated or purified antibody composition that selectively binds to an epitope-containing fragment of a SPANK polypeptide of claim 13.

Definitions

Before describing the invention in greater detail, the following definitions are set forth to illustrate and define the meaning and scope of the terms used to describe the invention herein.

As used interchangeably herein, the term "oligonucleotides", and "polynucleotides" include RNA, DNA, or RNA/DNA hybrid sequences of more than one nucleotide in either single chain or duplex form. The term "nucleotide" is used herein as an adjective to describe molecules comprising RNA, DNA, or RNA/DNA hybrid sequences of any length in single-stranded or duplex form. The term "nucleotide" is also used herein as a noun to refer to individual nucleotides or varieties of nucleotides, meaning a molecule, or individual unit in a larger nucleic acid molecule, comprising a purine or pyrimidine, a ribose or deoxyribose sugar moiety, and a phosphate group, or phosphodiester linkage in the case of nucleotides within an oligonucleotide or polynucleotide. In addition, the term "nucleotide" is also used herein to encompass "modified nucleotides" which comprise at least one modification, including for example: (a) an alternative linking group, (b) an analogous form of purine, (c) an analogous form of pyrimidine, or (d) an analogous sugar. For examples of analogous linking groups, purines, pyrimidines, and sugars see for example PCT publication No. WO 95/04064. The polynucleotide sequences of the invention may be prepared by any known method, including synthetic, recombinant, *ex vivo* generation, or a combination thereof, as well as utilizing any purification methods known in the art.

As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. Purification of starting material or natural material of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. As an example, purification from 0.1 % concentration to 10 % concentration is two orders of magnitude.

To illustrate, individual cDNA clones isolated from a cDNA library have been conventionally purified to electrophoretic homogeneity. The sequences obtained from these clones could not be obtained directly either from the library or from total human DNA. The cDNA clones are not naturally occurring as such, but rather are obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). The conversion of mRNA into a cDNA library involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection. Thus, creating a cDNA library from messenger RNA and subsequently isolating individual clones from that library results in an approximately 10^4-10^6 fold purification of the native message.

The term "purified" is further used herein to describe a polypeptide or polynucleotide of the invention which has been separated from other compounds including, but not limited to, polypeptides or polynucleotides, carbohydrates, lipids, etc. The term "purified" may be used to specify the separation of monomeric polypeptides of the invention from oligomeric forms such as homo- or hetero- dimers, trimers, etc. The term "purified" may also be used to specify the separation of covalently closed polynucleotides from linear polynucleotides. A polynucleotide is

substantially pure when at least about 50%, preferably 60 to 75% of a sample exhibits a single polynucleotide sequence and conformation (linear versus covalently closed). A substantially pure polypeptide or polynucleotide typically comprises about 50%, preferably 60 to 90% weight/weight of a polypeptide or polynucleotide sample, respectively, more usually about 95%, and preferably is over about 99% pure. Polypeptide and polynucleotide purity, or homogeneity, is indicated by a number of means well known in the art, such as agarose or polyacrylamide gel electrophoresis of a sample, followed by visualizing a single band upon staining the gel. For certain purposes higher resolution can be provided by using HPLC or other means well known in the art.

As an alternative embodiment, purification of the polypeptides and polynucleotides of the present invention may be expressed as "at least" a percent purity relative to heterologous polypeptides and polynucleotides (DNA, RNA or both). As a preferred embodiment, the polypeptides and polynucleotides of the present invention are at least; 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 96%, 98%, 99%, or 100% pure relative to heterologous polypeptides and polynucleotides, respectively. As a further preferred embodiment the polypeptides and polynucleotides have a purity ranging from any number, to the thousandth position, between 90% and 100% (*e.g.*, a polypeptide or polynucleotide at least 99.995% pure) relative to either heterologous polypeptides or polynucleotides, respectively, or as a weight/weight ratio relative to all compounds and molecules other than those existing in the carrier. Each number representing a percent purity, to the thousandth position, may be claimed as individual species of purity.

The term "isolated" requires that the material be removed from its original environment (*e.g.*, the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotide could be part of a vector and/or such polynucleotide or polypeptide could be part of a composition, and still be isolated in that the vector or composition is not part of its natural environment.

Specifically excluded from the definition of "isolated" are: naturally-occurring chromosomes (such as chromosome spreads), artificial chromosome libraries, genomic libraries, and cDNA libraries that exist either as an *in vitro* nucleic acid preparation or as a transfected/transformed host cell preparation, wherein the host cells are either an *in vitro* heterogeneous preparation or plated as a heterogeneous population of single colonies. Also specifically excluded are the above libraries wherein a specified sequence makes up less than 5% of the number of nucleic acid inserts in the vector molecules. Further specifically excluded are whole cell genomic DNA or whole cell RNA preparations (including said whole cell preparations which are mechanically sheared or enzymaticly digested). Further specifically excluded are the above whole cell preparations as either an *in vitro* preparation or as a heterogeneous mixture separated by electrophoresis (including blot transfers of the same) wherein the polynucleotide of the invention has not further been separated from the heterologous polynucleotides in the electrophoresis medium (*e.g.*, further separating by excising a single band from a heterogeneous band population in an agarose gel or nylon blot).

Throughout the present specification, the expression "nucleotide sequence" may be employed to designate either a polynucleotide or a nucleic acid. More precisely, the expression "nucleotide sequence" encompasses the nucleic material itself and is thus not restricted to the sequence information (*i.e.* the succession of letters designating the four bases) that biochemically characterizes a specific DNA or RNA molecule.

A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell required to initiate the specific transcription of a gene.

A sequence which is "operably linked" to a regulatory sequence such as a promoter means that said regulatory element is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the nucleic acid of interest.

As used herein, the term "operably linked" refers to a linkage of polynucleotide elements in a functional relationship. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the coding sequence. More precisely, two DNA molecules (such as a polynucleotide containing a promoter region and a polynucleotide encoding a desired polypeptide or polynucleotide) are said to be "operably linked" if the nature of the linkage between the two polynucleotides does not (1) result in the introduction of a frame-shift mutation or (2) interfere with the ability of the polynucleotide containing the promoter to direct the transcription of the coding polynucleotide.

The term "primer" denotes a specific oligonucleotide sequence that is complementary to a target nucleotide sequence and used to hybridize to the target nucleotide sequence. A primer serves as an initiation point for nucleotide polymerization catalyzed by DNA polymerase, RNA polymerase, or reverse transcriptase.

The term "probe" denotes a defined nucleic acid segment (or nucleotide analog segment, *e.g.*, PNA as defined hereinbelow) which can be used to identify a specific polynucleotide sequence present in a sample, said nucleic acid segment comprising a nucleotide sequence complementary to the specific polynucleotide sequence to be identified.

The terms "polynucleotide construct" and "recombinant polynucleotide" are used interchangeably herein to refer to linear or circular polynucleotides that have been artificially designed and which comprise at least two nucleotide sequences that are not found as contiguous nucleotide sequences in their initial natural environment.

The term "polypeptide" refers to a polymer of amino acids without regard to the length of the polymer. Thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not specify or exclude post-expression modifications of polypeptides. For example, polypeptides that include the covalent attachment of glycosyl groups, acetyl groups, phosphate groups, lipid groups and the like are expressly encompassed by the term polypeptide. Also included within the definition are polypeptides which contain one or more analogs of an amino acid (including, for example, non-naturally occurring amino acids, amino acids which only occur naturally in an unrelated biological system, modified amino acids from mammalian systems etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

The term "recombinant polypeptide" is used herein to refer to polypeptides that have been artificially designed, and that comprise at least two polypeptide sequences that are not found as contiguous polypeptide sequences in their initial natural environment, or to refer to polypeptides that have been expressed from a recombinant polynucleotide.

As used herein, the term "non-human animal" refers to any non-human vertebrate, birds and more usually mammals, preferably primates, farm animals such as swine, goats, sheep, donkeys, and horses, rabbits or rodents, more preferably rats or mice. As used herein, the term "animal" is used to refer to any vertebrate, preferably a mammal. Both the terms "animal" and "mammal" expressly embrace human subjects unless preceded with the term "non-human".

The term "disease involving the partitioning of dietary lipids between the liver and peripheral tissues " particularly refers to obesity and obesity related disorders such as obesity-related atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes.

The term "agent acting on the partitioning of dietary lipids between the liver and peripheral tissues" refers to a drug or a compound modulating the activity of SPANK or the partitioning of dietary lipids to the liver, reducing food intake in obese individuals, reducing the levels of free fatty acids in obese individuals, decreasing the body weight of obese individuals, or treating an obesity related condition selected from the group consisting of obesity-related

atherosclerosis, obesity-related insulin resistance, obesity-related hypertension, microangiopathic lesions resulting from obesity-related Type II diabetes, ocular lesions caused by microangiopathy in obese individuals with Type II diabetes, and renal lesions caused by microangiopathy in obese individuals with Type II diabetes.

The terms "response to an agent acting on the partitioning of dietary lipids between the liver and peripheral tissues " refer to drug efficacy, including but not limited to ability to metabolize a compound, to the ability to convert a pro-drug to an active drug, and to the pharmacokinetics (absorption, distribution, elimination) and the pharmacodynamics (receptor-related) of a drug in an individual.

The terms "side effects to an agent acting on the partitioning of dietary lipids between the liver and peripheral tissues " refer to adverse effects of therapy resulting from extensions of the principal pharmacological action of the drug or to idiosyncratic adverse reactions resulting from an interaction of the drug with unique host factors. "Side effects to an agent acting on the partitioning of dietary lipids between the liver and peripheral tissues " include, but are not limited to, adverse reactions such as dermatologic, hematologic or hepatologic toxicities and further includes gastric and intestinal ulceration, disturbance in platelet function, renal injury, nephritis, vasomotor rhinitis with profuse watery secretions, angioneurotic edema, generalized urticaria, and bronchial asthma to laryngeal edema and bronchoconstriction, hypotension, and shock.

The term "upstream" is used herein to refer to a location that is toward the 5' end of the polynucleotide from a specific reference point.

The terms "base paired" and "Watson & Crick base paired" are used interchangeably herein to refer to nucleotides which can be hydrogen bonded to one another be virtue of their sequence identities in a manner like that found in double-helical DNA with thymine or uracil residues linked to adenine residues by two hydrogen bonds and cytosine and guanine residues linked by three hydrogen bonds (See Stryer, L., Biochemistry, 4th edition, 1995).

The terms "complementary" or "complement thereof" are used herein to refer to the sequences of polynucleotides which is capable of forming Watson & Crick base pairing with another specified polynucleotide throughout the entirety of the complementary region. For the purpose of the present invention, a first polynucleotide is deemed to be complementary to a second polynucleotide when each base in the first polynucleotide is paired with its complementary base. Complementary bases are, generally, A and T (or A and U), or C and G. "Complement" is used herein as a synonym from "complementary polynucleotide", "complementary nucleic acid" and "complementary nucleotide sequence". These terms are applied to pairs of polynucleotides based solely upon their sequences and not any particular set of conditions under which the two polynucleotides would actually bind.

By "aberrant" is meant a change from the type or level of activity seen in normal cells, tissues, or individuals, or seen previously in the cell, tissue, or individual prior to the onset of the illness. The term "preventing" as used herein refers to administering a compound prior to the onset of clinical symptoms of a disease or conditions so as to prevent a physical manifestation of disease. The term "treating" as used herein refers to administering a compound after the onset of clinical symptoms.

The term "obesity-related diseases and disorders" as used herein refers to any disease or disorder or condition that is related to obesity, which in its extreme form includes individuals with a BMI of 40. "Obesity-related diseases and disorders" can also include, sometimes preferentially, diseases and disorders in which there are modulations in body mass. By "modulations in body mass" is meant those fluctuations in body weight (either increases or decreases) that may not be considered clinical obesity, including, but not limited to, changes of 5 or 10, or 20 to 30 or even 50 pounds, depending on the size of the individual. "Modulations in body mass" is also meant to encompass, in some circumstances, more localized increases in adipose tissue. for example, gains or losses specifically around the waist or hips, or around the hips and thighs, for example. These localized gains or losses of adipose tissue can be identified by increases or decreases in waist or hip size, for example.

The term "individual or patient" as used herein refers to a mammal, including animals, preferably mice, rats, dogs, cattle, sheep, or primates, most preferably humans that are in need of treatment. The term "in need of such treatment" as used herein refers to a judgment made by a care giver (physician, nurse, nurse practitioner, for example) in the case of humans that a patient or individual would benefit from or requires treatement. This judgment is made based on a variety of factors that are in the realm of a medical care giver's expertise, but that include the knowledge that the patient is ill, or will be ill, as the result of a condition that is treatable by the compounds of the invention. The term "patient or individual" can also refer to a perceived need to reduce body mass. The term "perceives a need" refers to changes in body mass that are typically not considered clinically obese, although could also include clinical obesity.

The terms "percentage of sequence identity" and "percentage homology" are used interchangeably herein to refer to comparisons among polynucleotides and polypeptides, and are determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Homology is evaluated using any of the variety of sequence comparison algorithms and programs known in the art. Such algorithms and programs include, but are by no means limited to, TBLASTN, BLASTP, FASTA, TFASTA, and CLUSTALW (Pearson and Lipman, 1988; Altschul *et al.*, 1990; Thompson *et al.*, 1994; Higgins *et al.*, 1996; Altschul *et al.*, 1990; Altschul *et al.*, 1993). In a particularly preferred embodiment, protein and nucleic acid sequence homologies are evaluated using the Basic Local Alignment Search Tool ("BLAST") which is well known in the art (see, *e.g.*, Karlin and Altschul, 1990; Altschul *et al.*, 1990, 1993, 1997). In particular, five specific BLAST programs are used to perform the following task:

(1) BLASTP and BLAST3 compare an amino acid query sequence against a protein sequence database;

(2) BLASTN compares a nucleotide query sequence against a nucleotide sequence database;

(3) BLASTX compares the six-frame conceptual translation products of a query nucleotide sequence (both strands) against a protein sequence database;

(4) TBLASTN compares a query protein sequence against a nucleotide sequence database translated in all six reading frames (both strands); and

(5) TBLASTX compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database.

The BLAST programs identify homologous sequences by identifying similar segments, which are referred to herein as "high-scoring segment pairs," between a query amino or nucleic acid sequence and a test sequence which is preferably obtained from a protein or nucleic acid sequence database. High-scoring segment pairs are preferably identified (i.e., aligned) by means of a scoring matrix, many of which are known in the art. Preferably, the scoring matrix used is the BLOSUM62 matrix (Gonnet *et al.*, 1992; Henikoff and Henikoff, 1993). Less preferably, the PAM or PAM250 matrices may also be used (see, *e.g.*, Schwartz and Dayhoff, eds., 1978). The BLAST programs evaluate the statistical significance of all high-scoring segment pairs identified, and preferably selects those segments which satisfy a user-specified threshold of significance, such as a user-specified percent homology. Preferably, the statistical significance of a high-scoring segment pair is evaluated using the statistical significance formula of Karlin (see, *e.g.*, Karlin and Altschul, 1990).

By way of example and not limitation, procedures using conditions of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65 °C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/mL denatured salmon sperm DNA. Filters are hybridized for 48 h at 65 °C, the preferred hybridization temperature, in prehybridization mixture containing 100 μg/mL denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Alternatively, the hybridization step can be performed at 65 °C in the presence of SSC buffer, 1 x SSC corresponding to 0.15M NaCl and 0.05 M Na citrate. Subsequently, filter washes can be done at 37 °C for 1 h in a solution containing 2 x SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA, followed by a wash in 0.1 X SSC at 50 °C for 45 min. Alternatively, filter washes can be performed in a solution containing 2 x SSC and 0.1% SDS, or 0.5 x SSC and 0.1% SDS, or 0.1 x SSC and 0.1% SDS at 68 °C for 15 minute intervals. Following the wash steps, the hybridized probes are detectable by autoradiography. Other conditions of high stringency may also be used and are well known in the art (Sambrook *et al.*, 1989 and Ausubel *et al.*, 1989, both of which are hereby incorporated herein in their entirety including any drawings, figures, or tables. These hybridization conditions are suitable for a nucleic acid molecule of about 20 nucleotides in length. The hybridization conditions described can be adapted according to the length of the desired nucleic acid, following techniques well known to the one skilled in the art. The suitable hybridization conditions may, for example, be adapted according to Hames and Higgins (1985) or Sambrook *et al.* (1989).

As used herein, the term "antibody" refers to a polypeptide or group of polypeptides which are comprised of at least one binding domain, where an antibody binding domain is formed from the folding of variable domains of an antibody molecule to form three-dimensional binding spaces with an internal surface shape and charge distribution complementary to the features of an antigenic determinant of an antigen, that allows an immunological reaction with the antigen. Antibodies include recombinant proteins comprising the binding domains, as wells as fragments, including Fab, Fab', F(ab)$_2$, and F(ab')$_2$ fragments.

As used herein, an "antigenic determinant" is the portion of an antigen molecule, in this case a SPANK polypeptide, that determines the specificity of the antigen-antibody reaction. An "epitope" refers to an antigenic determinant of a polypeptide. An epitope can comprise as few as 3 amino acids in a spatial conformation that is unique to the epitope. generally an epitope consists of at least 6 such amino acids, and more usually at least 8-10 such amino acids. Methods for determining the amino acids which make up an epitope include x-ray crystallography, 2-dimensional nuclear magnetic resonance, and epitope mapping *e.g.* the Pepscan method described by H. Mario Geysen *et al.* 1984. Proc. Natl. Acad. Sci. U.S.A. 81:3998-4002; PCT Publication No. WO 84/03564; and PCT Publication No. WO 84/03506.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of SPANK in which an ANK domain ; a SAM domain; and a PARP catalytic domain (poly(ADP-ribose) polymerase) and two additional domains are illustrated. Within the ANK domain, every fourth ankyrin repeat is interrupted by an insert (represented by an oval). The ANK domain binds to IRAP, thus tethering GLUT4 vesicles to cytoskeletal myosin in the perinuclear region.

Figures 2A-B are schematic representations of four mechanisms by which SPANK may regulate GLUT4 targeting. Figure 2A represents tethering of GLUT4 vesicles by SPANK. Figure 2B represents SPANK as a scaffold for GLUT4 vesicles and signaling molecules. Figure 2C represents SPANK as a signal transducer immediately upstream of GLUT4 vesicles. Figure 2D represents SPANK as a carrier of GLUT4 vesicles.

Figure 3 is the amino acid sequence of human SPANK protein (SEQ ID NO.:1).

Figures 4A and 4B are the nucleic acid sequence (6123 nucleotides) of human SPANK cDNA (SEQ ID NO.: 2), in which the start codon and the stop codon are shown in bold lettering.

Figure 5A is a partial nucleic acid sequence of mouse SPANK cDNA (SEQ ID NO.: 3), in which the start codon is shown in bold lettering

Figure 5B is a partial amino acid sequence of mouse SPANK protein (SEQ ID NO.: 4) aligned with human SPANK protein

Figure 6 is a partial nucleic acid sequence of rat SPANK cDNA (SEQ ID NO.: 5).

Figure 7 is a partial amino acid sequence of rat SPANK protein (SEQ ID NO.:6).

Figure 8 is an alignment of human SPANK and Tankyrase proteins.

DETAILED DESCRIPTION OF THE INVENTION

I. SPANK Polypeptides of the Invention

The present invention relates *inter alia* to a cytosolic protein, SPANK, that is an effector in the insulin signaling pathway in eukaryotic cells (*e.g.*, human, mouse, rat, other mammalian cells). SPANK has been shown to be abundantly expressed in insulin-sensitive tissues and to be induced during adipocyte differentiation. SPANK is targeted near the Golgi apparatus and co-localizes with GLUT4 in the perinuclear region of adipocytes. Brief treatment of adipocytes with niacinamide, which can inhibit the PARP activity of SPANK, results in significant impairment of insulin-induced GLUT4 translocation. SPANK is unique in that it has been shown to physically interact with a component of GLUT4 vesicles, IRAP (also referred to as VP160 and VP165), a major constituent of GLUT4 vesicles.

Isolated SPANK protein of the present invention is eukaryotic, particularly mammalian (*e.g.*, human, mouse, rat, pig, cow, goat, sheep). The amino acid sequence of human SPANK (Fig. 3; SEQ ID NO:1), as well as a partial amino acid sequence of mouse SPANK protein (Fig. 5B; SEQ ID NO:4) and a partial amino acid sequence of rat SPANK protein (Fig. 7; SEQ ID NO:6), are presented herein. SPANK may comprise in the following order: an ANK domain, a SAM domain and a PARP catalytic domain. As represented in Figure 1, the three domains are separated by two intervening regions, a first region between the C-terminal end of the ANK domain and the N-terminal end of the SAM domain and a second region between the C-terminal end of the SAM domain and the N-terminal end of the PARP domain. In addition, SPANK may include an amino terminal region and short C-terminal region as shown in Fig. 1.

The ANK domain of SPANK (approximately amino acid residues 43 to 785) comprises 20 copies of the ANK repeat (20 ankyrin repeats), a motif previously identified in ankyrin that binds several integral membrane proteins. The ANK domain of SPANK binds to the cytosolic domain of IRAP that contains the SPANK-binding motif, RQSPDG (amino acid residues 96-101 of IRAP), in which R represents arginine; Q, glutamine; S, serine ; P, proline; D, aspartic acid; and G, glycine).

The SAM domain of SPANK (approximately amino acid residues 870 to 935) is a structural motif found in many signaling molecules. This motif can mediate protein - protein interaction, causing both homodimerization and heterodimerization of proteins.

The PARP domain of SPANK (approximately amino acid residues 1023 to 1161) is a catalytic domain whose activity makes it possible for SPANK to poly (ADP-ribosyl)ate itself and other substrates *in vitro*.

In addition to these three domains, SPANK comprises an amino terminal domain (approximately amino acid residues 1 to 42), a short C-terminal domain (approximately amino acid residues 1162-1166), and two intervening domains (approximately amino acid residues 786 to 869 and approximately amino acid residues 936 to 1022) represented in Figure 1.

The invention also relates to SPANK fragments, such as fragments that comprise amino acid residues that bind a six amino acid region (RQSPDG) within the cytosolic domain of IRAP and particularly fragments of the ANK domain that bind the six amino acid region. The invention also relates to the SPANK fragment lacking the ANK domain, which is sufficient to bind myosin (approximately amino acid residues 786 to 1166), as represented in Figure 1.

SPANK protein of the present invention can comprise an amino acid sequence presented herein, such as SEQ ID NO.:1; SEQ ID NO.: 4; or SEQ ID NO.: 6 or an amino acid sequence sufficiently similar to one of these sequences that the protein exhibits IRAP binding activity and participates in GLUT4 translocation (from the interior of a cell to the cell membrane) in response to insulin stimulation of the cell. In one embodiment of the invention, SPANK protein exhibits at least 82% identity to an amino acid sequence presented herein. Additional embodiments are proteins at least 85% identical to a sequence presented herein, at least 90% or 95% identical to an amino acid sequence or at least 97-98% identical to an amino acid sequence presented herein.

A protein of the present invention can comprise an ANK domain, a SAM domain and a PARP catalytic domain. The two intervening domains positioned, respectively, between the C-terminal end of the ANK domain and the N-terminal end of the SAM domain and between the C-terminal end of the SAM domain and the N-terminal end of the PARP domain, can comprise a sequence presented herein or can be different from those presented herein, provided that SPANK activity is retained. For example, the intervening domains can differ from the sequences presented herein by one or more amino acid residues. They can be, for example, linker sequences of appropriate composition and length that join the three SPANK domains (ANK, SAM, PARP) in such a manner that SPANK activity is retained.

Similarly, SPANK fragments of the present invention can comprise a fragment of a protein whose sequence is presented herein, such as a fragment that binds IRAP, or can be fragments in which the amino acid residues differ by at least one addition, deletion, substitution or modification from the sequences represented herein, provided that the desired function (*e.g.*, IRAP binding) is retained.

Proteins and protein fragments of the present invention can be obtained (isolated) from sources in which they occur in nature (*e.g.*, adipocytes, muscle cells), can be produced using recombinant DNA technology (*e.g.*, by expressing DNA encoding SPANK protein in an appropriate host cell) or can be made using chemical synthetic methods.

The invention relates to SPANK polypeptides as well as to variants, fragments, analogs and derivatives of the SPANK polypeptides described herein, including modified SPANK polypeptides. By SPANK as used herein is meant a polypeptide such as SEQ ID NO.:1; SEQ ID NO.: 4; or SEQ ID NO.: 6, or fragments, or variants thereof. "SPANK" further includes the full-length polypeptides of which SEQ ID NO:4 and SEQ ID NO:6 comprise only a fragment. The full-length polypeptides can be obtained by methods similar to those described herein for SEQ ID NO: 1, as well as other methods well-known in the art.

A SPANK polypeptide has one or more of the following activities *in vitro*, *in vivo*, or in humans: binding to IRAP, binding to myosin, and PARP activity. These fragments or variants preferably are as active as the intact SPANK in an *in vitro* or an *in vivo* assay or in humans, more preferably are more active than the intact SPANK in any one of these assays, and most preferably are significantly more active than the intact SPANK in any one of these assays. Alternatively, these fragments or variants are less active than the intact SPANK in any one of these assays, and preferably are significantly less active than the intact SPANK in any one of these assays. The SPANK fragments and variants may also retain some, but not all of the described SPANK activities.

A "fragment" of a SPANK polypeptide as used herein is a polypeptide having a sequence that is entirely the same as part, but not all, of a given polypeptide sequence, preferably a SPANK polypeptide, and variants thereof. Such fragments may be "free-standing", *i.e.* not part of or fused to other polypeptides, or they may form a part or

region of a longer polypeptide. In addition, a single larger polypeptide may comprise several SPANK fragments. As representative examples of SPANK polypeptide fragments of the invention, there may be mentioned those which have from 5, 6, 7, 8, 9 or 10 to 15, 10 to 20, 15 to 40, 30 to 55, 40 to 70, 60 to 95, 80 to 130, 90 to 150, 140 to 200, 180 to 240, 220 to 280, 260 to 320, 300 to 360, 340 to 400, 380 to 440, 420 to 500, 480 to 540, 520 to 580, 560 to 620, 680 to 740, 720 to 780, 840 to 900, 880 to 960, 940 to 1020, 1000 to 1080, 1060 to 1140, 1120 to 1166 amino acids long. However, SPANK polypeptide fragments of any length from 108 to 1166 amino acids of SEQ ID NO:1 (*i.e.* 108, 109, 110, 111, 112, or 113, etc), or the comparable sequences for mouse SPANK as shown in Fig. 5B are specifically contemplated. Other preferred fragments are described below.

The term "activity" as used herein refers to a measurable result of the interaction of molecules. For example, a measurable SPANK activity includes binding to IRAP, binding to myosin, or PARP activity. Methods for detecting these activities are described in the Examples, and other comparable methods well-known in the art can also be used. These assays and other comparable assays can be used to determine/identify SPANK variants, analogs, derivatives, or fragments that retain all or some SPANK activities, or that are more or less active than the intact SPANK for some or all of the activities.

The term "retains the same activity" as used herein refers to a comparison of a measurable activity of SPANK polypeptides to an appropriate control, such as the intact reference SPANK polypeptide described herein (SEQ ID NO:1). For example, one or more of the SPANK activities of IRAP-binding, myosin-binding, and PARP activity could be compared among some variants or fragments as compared to the exemplary intact SPANK polypeptide described herein. Preferably, "the same activity is retained" when a change is undetectable by methods described herein, or other methods known in the art, or the change is within the error associated with the experimental method, or the change is less than an increase or decrease of 5%, or less than an increase10% or less than an increase or decrease of 25% compared to the level of activity in the presence of intact reference SPANK.

The term "more active" as used herein refers to a measurable increase in an activity of SPANK polypeptides as compared to an appropriate control, such as the intact reference SPANK polypeptide described herein. For example, one or more of the SPANK activities of IRAP-binding, myosin-binding, and PARP activity might be increased in some variants or fragments as compared to the reference SPANK polypeptide described herein. Preferably, an increase in activity is at least 25%, at least 50%, at least 75%, or at least 100% compared to the level of activity in the presence of reference SPANK.

Similarly, the term "less active" as used herein refers to a measurable decrease in an activity of SPANK polypeptides as compared to an appropriate control, such as the intact reference SPANK polypeptide described herein. For example, one or more of the SPANK activities of IRAP-binding, myosin-binding, and PARP activity might be decreased in some variants or fragments as compared to the reference intact SPANK polypeptide described herein. Preferably, a decrease in activity is at least 25%, at least 50%, at least 75%, or at least 95% compared to the level of activity in the presence of reference SPANK.

Preferred embodiments of the invention feature SPANK polypeptide that consists of SEQ ID NO.:1; SEQ ID NO.: 4; or SEQ ID NO.: 6, or variants, fragments, analogs, or derivatives thereof. Preferable embodiments include polypeptides that comprise or polypeptides that consist of amino acids 43 to 785 of SEQ ID NO:1; amino acids 870 to 935 of SEQ ID NO:1; amino acids 1023 to 1161 of SEQ ID NO:1; amino acids 1 to 42 of SEQ ID NO:1; amino acids 1162-1166 of SEQ ID NO:1; amino acids 786 to 869 of SEQ ID NO:1; amino acids 936 to 1022 of SEQ ID NO:1; and amino acids 786 to 1166 of SEQ ID NO:1; or the equivalent amino acids of mouse SPANK as seen in the alignment between human and mouse SPANK shown in Fig. 5B. Preferably the SPANK polypeptide is human, rat, or mouse, but most preferably human.

Variant SPANK polypeptides of the invention may be 1) ones in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue and such substituted amino acid residue may or may not be one encoded by the genetic code, or 2) ones in which one or more of the amino acid residues includes a substituent group, or 3) ones in which a modified SPANK polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or 4) ones in which the additional amino acids are fused to modify a SPANK polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the modified SPANK polypeptide or a pre-protein sequence. Such variants are deemed to be within the scope of those skilled in the art. The retention of the desired activity (and thus desired SPANK polypeptides) can be determined using the assays described in Examples 1-5 and 7-9 or other assays known in the art that achieve the same result.

Amino acid changes present in a variant polypeptide may be non-conservative amino acid changes but more preferably are conservative amino acid changes. In cases where there are one or more amino acid changes, preferred SPANK polypeptides include those that retain the same activities and activity levels as the reference SPANK polypeptide sequence, as well as those where the level of one or more activities is increased or decreased, as long as at least one SPANK activity is retained. Assays for determining SPANK polypeptide activities of the invention are described herein in the Examples (1-5 and 7-9) in more detail, but include IRAP binding, myosin binding and PARP activity.

In the case of an amino acid substitution in the amino acid sequence of a SPANK polypeptide, one or several amino acids can be replaced by "equivalent" amino acids. The expression "equivalent" amino acid is used herein to designate any amino acid that may be substituted for one of the amino acids having similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. generally, the following groups of amino acids represent equivalent changes: (1) Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr; (2) Cys, Ser, Tyr, Thr; (3) Val, Ile, Leu, Met, Ala, Phe; (4) Lys, Arg, His; (5) Phe, Tyr, Trp, His.

A specific embodiment of a modified SPANK polypeptide, includes, but is not limited to, a polypeptide that is resistant to proteolysis, such as a peptide in which the -CONH- peptide bond is modified and replaced by a (CH_2NH) reduced bond, a (NHCO) retro inverso bond, a (CH_2-O) methylene-oxy bond, a (CH_2-S) thiomethylene bond, a (CH_2CH_2) carba bond, a (CO-CH_2) cetomethylene bond, a ($CHOH$-CH_2) hydroxyethylene bond), a (N-N) bound, a E-alcene bond or also a -CH=CH- bond. The invention also encompasses a SPANK polypeptide or a fragment or a variant thereof in which at least one peptide bond has been modified as described above.

In preferred embodiments, the invention features a variant of a SPANK polypeptide that is at least 82% identical to SPANK polypeptide sequences selected from the group consisting of SEQ ID NO: 1, SEQ ID NO:4, and SEQ ID NO:6. Preferably, the amino acid sequence is at least 85% identical, more preferably 90% identical, most preferably 95% identical and optionally 100% identical. Preferably the sequence is human, mouse, or rat, and most preferably human.

In yet other preferred embodiments, the invention features a variant of a SPANK polypeptide that comprises a 1166 contiguous amino acid sequence, wherein at least 911 of the 1166 amino acids are identical to amino acids of SEQ ID NO:1. Preferably, at least 925 of the 1166 amino acids are identical, at least 950 of the 1166 are identical, a least 975 of the 1166 are identical, at least 1000 of the 1166 are identical, at least 1125 of the 1166 are identical, at least 1150 of the 1166 are identical, and most preferably all of the amino acids are identical. Alternatively, the invention features a variant of a SPANK polypeptide that consists of a 1166 contiguous amino acid sequence, wherein at least 911 of the 1166 amino acids are identical to amino acids of SEQ ID NO:1. Preferably, at least 925 of the 1166 amino acids are identical, at least 950 of the 1166 are identical, a least 975 of the 1166 are identical, at least

1000 of the 1166 are identical, at least 1125 of the 1166 are identical, at least 1150 of the 1166 are identical, and most preferably all of the amino acids are identical. Preferably the sequence is human, mouse, or rat and most preferably human.

In other preferred embodiments, the SPANK polypeptide comprises a fragment or variant of SPANK that is useful to modulate an activity of SPANK *in vitro, in vivo,* or in humans. Preferably the activity that is modulated is selected from the group consisting of IRAP binding, myosin binding, GLUT4 translocation, insulin signaling, insulin resistance, and glucose sensitivity.

The term "modulation" of activity as used herein refers to a measurable change in an activity. Examples of such activities include, modulation of the IRAP binding, myosin binding, GLUT4 translocation, insulin signaling, insulin resistance, and glucose sensitivity. Modulation of an activity can be either an increase or a decrease in the activity, for example as the result of addition of exogenous SPANK polypeptides, to include fragments, analogs, derivatives, and variants. Exemplary assays are provided in Examples 1-5 and 7-9. These assays and other comparable assays can be used to determine/identify SPANK variants, analogs, derivatives, or fragments that modulate SPANK activities including, but not limited to, IRAP binding, myosin binding, GLUT4 translocation, insulin signaling, insulin resistance, and glucose sensitivity *in vitro* and *in vivo*.

The term "increase in activity" as used herein refers to the ability of SPANK polypeptides to increase an activity in some measurable way as compared to an appropriate control. As a result of the presence of SPANK, IRAP binding, myosin binding, GLUT4 translocation, insulin signaling, insulin resistance, and/or glucose sensitivity might increase, for example, as compared to controls in the absence of SPANK. Preferably, an increase in activity is at least 25%, at least 50%, at least 75%, or at least 100% compared to the level of activity in the absence of SPANK.

Similarly, the term "decrease in activity" as used herein refers to the ability of SPANK polypeptides to decrease an activity in some measurable way as compared to an appropriate control. For example, as a result of the presence of SPANK, IRAP binding, myosin binding, GLUT4 translocation, insulin signaling, insulin resistance, and/or glucose sensitivity might decrease for example, as compared to controls in the absence of SPANK. Preferably, a decrease in activity is at least 25%, at least 50%, at least 75%, or at least 100% compared to the level of activity in the absence of SPANK.

SPANK proteins are preferably isolated from human or mammalian tissue samples or expressed from human or mammalian genes. The SPANK polypeptides of the invention can be made using routine expression methods known in the art. The polynucleotide encoding the desired polypeptide, is ligated into an expression vector suitable for any convenient host. Both eukaryotic and prokaryotic host systems can be used in forming recombinant polypeptides, and a summary of some of the more common systems is given herein. The polypeptide is then isolated from lysed cells or from the culture medium and purified to the extent needed for its intended use. Purification is by any technique known in the art, for example, differential extraction, salt fractionation, chromatography, centrifugation, and the like. See, for example, Methods in Enzymology for a variety of methods for purifying proteins. In addition, shorter protein fragments can be produced by chemical synthesis. Alternatively the proteins of the invention are extracted from cells or tissues of humans or non-human animals. Methods for purifying proteins are known in the art, and include the use of detergents or chaotropic agents to disrupt particles followed by differential extraction and separation of the polypeptides by ion exchange chromatography, affinity chromatography, sedimentation according to density, and gel electrophoresis.

Any SPANK cDNA can be used to express SPANK proteins and polypeptides. The nucleic acid encoding the SPANK protein or polypeptide to be expressed is operably linked to a promoter in an expression vector using conventional cloning technology. The SPANK insert in the expression vector may comprise the full coding sequence for the SPANK protein or a portion thereof as described previously and herein.

The expression vector is any of the mammalian, yeast, insect or bacterial expression systems known in the art and described briefly herein. Commercially available vectors and expression systems are available from a variety of suppliers including Genetics Institute (Cambridge, MA), Stratagene (La Jolla, California), Promega (Madison, Wisconsin), and Invitrogen (San Diego, California). If desired, to enhance expression and facilitate proper protein folding, the codon context and codon pairing of the sequence is optimized for the particular expression organism in which the expression vector is introduced, as explained by Hatfield, *et al.*, U.S. Patent No. 5,082,767, the disclosures of which are incorporated by reference herein in their entirety.

In one embodiment, the entire coding sequence of the SPANK cDNA through the poly A signal of the cDNA are operably linked to a promoter in the expression vector. Alternatively, if the nucleic acid encoding a portion of the SPANK protein lacks a methionine to serve as the initiation site, an initiating methionine can be introduced next to the first codon of the nucleic acid using conventional techniques. Similarly, if the insert from the SPANK cDNA lacks a poly A signal, this sequence can be added to the construct by, for example, splicing out the Poly A signal from pSG5 (Stratagene) using BglI and SalI restriction endonuclease enzymes and incorporating it into the mammalian expression vector pXT1 (Stratagene). pXT1 contains the LTRs and a portion of the gag gene from Moloney Murine Leukemia Virus. The position of the LTRs in the construct allow efficient stable transfection. The vector includes the Herpes Simplex Thymidine Kinase promoter and the selectable neomycin gene.

The expressed protein can be purified using conventional purification techniques such as ammonium sulfate precipitation or chromatographic separation based on size or charge. The protein encoded by the nucleic acid insert may also be purified using standard immunochromatography techniques. In such procedures, a solution containing the expressed SPANK protein or portion thereof, such as a cell extract, is applied to a column having antibodies against the SPANK protein or portion thereof is attached to the chromatography matrix. The expressed protein is allowed to bind the immunochromatography column. Thereafter, the column is washed to remove non-specifically bound proteins. The specifically bound expressed protein is then released from the column and recovered using standard techniques.

To confirm expression of the SPANK protein or a portion thereof, the proteins expressed from host cells containing an expression vector containing an insert encoding the SPANK protein or a portion thereof can be compared to the proteins expressed in host cells containing the expression vector without an insert. The presence of a band in samples from cells containing the expression vector with an insert which is absent in samples from cells containing the expression vector without an insert indicates that the SPANK protein or a portion thereof is being expressed. Generally, the band will have the mobility expected for the SPANK protein or portion thereof. However, the band may have a mobility different than that expected as a result of modifications such as glycosylation, ubiquitination, or enzymatic cleavage.

Antibodies capable of specifically recognizing the expressed SPANK protein or a portion thereof are described below and in Example 10.

The nucleic acids encoding the SPANK protein or a portion thereof can also be incorporated into expression vectors designed for use in purification schemes employing chimeric polypeptides. In such strategies the nucleic acid encoding the SPANK protein or a portion thereof is inserted in frame with the gene encoding the other half of the chimera. The other half of the chimera is -globin or a nickel binding polypeptide encoding sequence. A chromatography matrix having antibody to -globin or nickel attached thereto is then used to purify the chimeric protein. Protease cleavage sites is engineered between the -globin gene or the nickel binding polypeptide and the SPANK protein or portion thereof. Thus, the two polypeptides of the chimera are separated from one another by protease digestion.

One useful expression vector for generating -globin chimeric proteins is pSG5 (Stratagene), which encodes rabbit -globin. Intron II of the rabbit -globin gene facilitates splicing of the expressed transcript, and the

polyadenylation signal incorporated into the construct increases the level of expression. These techniques are well known to those skilled in the art of molecular biology. Standard methods are published in methods texts such as Davis *et al.*, (1986) and many of the methods are available from Stratagene, Life Technologies, Inc., or Promega. Polypeptide may additionally be produced from the construct using in vitro translation systems such as the In vitro Express™ Translation Kit (Stratagene).

II. SPANK Polynucleotides of the Invention

This invention further relates to isolated SPANK- encoding DNA (isolated DNA that encodes a protein that binds IRAP) and particularly to isolated DNA that encodes SPANK protein that comprises the domains described herein. Specifically, isolated DNA of the present invention comprises DNA that encodes a SAM domain, a PARP catalytic domain, an ANK domain and two additional domains, one between the C-terminal end of the ANK domain and the N-terminal end of the SAM domain and one between the C-terminal end of the SAM domain and the N-terminal end of the PARP domain. SPANK may also comprise the amino terminal region or short C-terminal region as shown in Fig. 1. In addition, this invention encompasses isolated DNA that encodes SPANK fragments, such as isolated DNA that encodes SPANK fragments that comprise amino acid residues that bind the six amino acid residue region of IRAP described herein and particularly fragments of the ANK domain that do so.

Isolated SPANK-encoding polynucleotides of the present invention are eukaryotic, particularly mammalian (*e.g.*, human, mouse, rat, pig, cow, goat, sheep). The nucleic acid sequence of cDNA encoding human SPANK protein (Fig. 4A & 4B), a partial mouse SPANK cDNA sequence (Fig. 5A) and a partial nucleic acid sequence of rat SPANK DNA (Fig. 6) are presented herein. Isolated eukaryotic, particularly mammalian, DNA (*e.g.*, human, mouse, rat, pig, goat, cow, sheep, other mammalian DNA), which encodes a protein which binds IRAP and is an effector in the insulin signaling pathway in a cell is also the subject of this invention.

The isolated DNA comprises, for example, DNA selected from the group consisting of DNA having the nucleic acid sequence of SEQ ID NO.:2; DNA having a nucleic acid sequence which is at least 85% identical to the nucleic acid sequence of SEQ ID NO.:2 and encodes a protein which binds IRAP; DNA having a nucleic acid sequence which is at least 95% identical to the nucleic acid sequence of SEQ ID NO.:2 and encodes a protein which binds IRAP; DNA which encodes the amino acid sequence of SEQ ID NO.:1; DNA which hybridizes under high stringency conditions to SEQ ID NO.: 2 or to the complement of SEQ ID NO.:2; DNA comprising the nucleic acid sequence of SEQ ID NO.:3; DNA which encodes a protein comprising the amino acid sequence of SEQ ID NO.:4; DNA which hybridizes under high stringency conditions to SEQ ID NO.: 3 or to the complement of SEQ ID NO.: 3; DNA comprising the nucleic acid sequence of SEQ ID NO: 5 or the complement thereof; DNA which hybridizes under high stringency conditions to DNA comprising SEQ ID NO.: 5 or to the complement of DNA comprising SEQ ID NO.: 5; and DNA which encodes a protein comprising the amino acid sequence of SEQ ID NO.: 6.

Examples of stringency conditions are as follows: hybridization in ExpressHyb solution (Clontech) at 68 °C followed by three or more washes in 0.1 x SSC/0.1% SDS at 50 °C for one hour or the same conditions except that the washes are carried out at 55 °C.

Also the subject of this invention is isolated DNA (*e.g.*, human, mouse, rat, other mammal) encoding a protein which comprises the domains present in SPANK, binds IRAP and participates in GLUT4 translocation in cells stimulated by insulin. The domains are an ANK domain, a SAM domain, a PARP catalytic domain and two intervening domains as described herein.

Isolated DNA of the present invention can be obtained from sources in which it occurs in nature or can be produced by recombinant DNA techniques or synthesized (*e.g.*., using chemical synthetic techniques). As used herein, the term "isolated DNA" encompasses DNA from all such sources. DNA can be genomic DNA or cDNA.

Polynucleotides have been designed that encode SPANK polypeptides of the invention (Section I). The recombinant polynucleotides encoding SPANK polypeptides can be used in a variety of ways, including, but not limited to expressing the polypeptide in recombinant cells to facilitate purification of the polypeptide.

The invention relates to the polynucleotides encoding SPANK polypeptides described in the Examples (1-5 and 7-9), and variants and fragments thereof as described in Section I of the Detailed Description of the Invention.

In other preferred embodiments, variants of SPANK polynucleotides encoding SPANK polypeptides are envisioned. Variants of polynucleotides, as the term is used herein, are polynucleotides whose sequence differs from a reference polynucleotide. A variant of a polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical.

Variants of SPANK polynucleotides according to the invention may include, without being limited to, nucleotide sequences which are at least 90% (preferably at least 95%, more preferably at least 99%, and most preferably at least 99.5%) identical to a polynucleotide that encodes a SPANK polypeptide of the invention, or to any polynucleotide fragment of at least 8 (preferably at least 15, at least 25, at least 45, at least 100, 200, 400, 700, 1000, 1500, 2000, 2500, or 3000) consecutive nucleotides of a polynucleotide that encodes a polypeptide of the invention.

Nucleotide changes present in a variant polynucleotide are preferably silent, which means that they do not alter the amino acids encoded by the polynucleotide. However, nucleotide changes may also result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence. The substitutions, deletions or additions may involve one or more nucleotides. Alterations in SPANK coding regions of the invention may produce conservative or non-conservative amino acid substitutions, deletions or additions in the encoded protein.

In cases where the nucleotide substitutions result in one or more amino acid changes, preferred SPANK polypeptides include those that retain the same activities and activity levels as the SPANK polypeptide encoded by the polynucleotide sequence described herein, as well as those where the level of one or more activities is increased or alternatively decreased. SPANK polypeptide activities of the invention are described herein in the Examples in more detail (1-5 and 7-9), but include IRAP binding, myosin binding, and PARP activity. Examples of assays to determine the presence or absence of specific SPANK activities and the level of the activity(s) are also described herein in Examples 1-5 and 7-9.

A polynucleotide fragment is a polynucleotide having a sequence that is the same as part, but not all, of a given nucleotide sequence, preferably the nucleotide sequence encoding a SPANK polypeptide and variants thereof as described above, and the complements of these polynucleotides. Preferably the polynucleotide fragment encodes a SPANK polypeptide fragment described previously in Section I, and complements thereof. Such fragments may be "free-standing", *i.e.* not part of or fused to other polynucleotides, or they may form a part or region of another polynucleotide. In addition, a single larger polynucleotide may comprise more than one fragment of SPANK.

Optionally, such fragments may consist of a contiguous span which ranges in length from 18, 51, 75, 102, or 141, to 201, 252, 300, 351, 402, 432, 700, 1000, 1500, 2000, 2500, or 3000 nucleotides, or be specified as 18, 51, 75, 102, 141, 201, 252, 300, 351, 402, 452, 502, 552, 602, 652, 702, 752, 802, 852, 902, 952, 1002, 1052, 1102, 1152, 1202, 1252, 1302, 1352, 1402, 1452, 1502, 1552, 1602, 1652, 1702, 1752, 1802, 1852, 1902, 1952, 2002, 2052, 2102, 2152, 2202, 2252, 2302, 2352, 2402, 2452, 2502, 2552, 2602, 2652, 2702, 2752,

2802, 2852, 290, 2952, 3002, 3052, 3102, 3152, 3202, 3252, 3302, 3352, 3402, 3452 or 3498 nucleotides in length.

A preferred embodiment of the invention includes isolated, purified, or recombinant polynucleotides consisting of a contiguous span of at least 18, 51, 75, 102, 141, 201, 252, 300, 351, 402, 452, 502, 552, 602, 652, 702, 752, 802, 852, 902, 952, 1002, 1052, 1102, 1152, 1202, 1252, 1302, 1352, 1402, 1452, 1502, 1552, 1602, 1652, 1702, 1752, 1802, 1852, 1902, 1952, 2002, 2052, 2102, 2152, 2202, 2252, 2302, 2352, 2402, 2452, 2502, 2552, 2602, 2652, 2702, 2752, 2802, 2852, 290, 2952, 3002, 3052, 3102, 3152, 3202, 3252, 3302, 3352, 3402, 3452 or 3498 nucleotides encoding a SPANK polypeptide of the invention, or the complements thereof, wherein said contiguous span encodes a fragment of SPANK that retains one or more of the activities and activity levels as the SPANK polypeptide described herein and encoded by the polynucleotide sequence described herein, or encodes a fragment of SPANK wherein the level of one or more activities is increased or decreased, as described above.

An additional preferred embodiment of the invention includes isolated, purified, or recombinant polynucleotides consisting of a contiguous span of any one of 8 to 1498 nucleotides (intended to encompass polynucleotides of every length between 8 and 1498, *i.e.* 8, 9, 10, 11, 12, 13, etc.) of a SPANK polypeptide of the invention, or the variants, or the complements thereof, wherein said contiguous span encodes a fragment of SPANK that retains the same activities and activity levels as SPANK polypeptide encoded by the SPANK polynucleotide sequence described herein, or encodes a fragment of SPANK where the level of one or more activities is increased, or is decreased as described above. Any of the above-described fragments may be comprised within a larger polynucleotide fragment.

Another object of the invention relates to purified, isolated or recombinant nucleic acids comprising a polynucleotide that hybridizes, under the stringent hybridization conditions defined herein, with a polynucleotide of SEQ ID No 2, SEQ ID NO: 3, or SEQ ID NO: 5, or a sequence complementary thereto or a variant thereof or a biologically active fragment thereof.

The polynucleotides described herein may further comprise nucleic acid fragments of any size and sequence flanking them or on either side, whether of genomic or other origin.

III. Recombinant Vectors of the Invention

The term "vector" is used herein to designate either a circular or a linear DNA or RNA molecule, that is either double-stranded or single-stranded, and that comprises at least one polynucleotide of interest that is sought to be transferred in a cell host or in a unicellular or multicellular host organism.

The present invention relates to recombinant vectors comprising any one of the polynucleotides described herein.

The present invention encompasses a family of recombinant vectors that comprise polynucleotides encoding SPANK polypeptides of the invention.

In a first preferred embodiment, a recombinant vector of the invention is used to amplify the inserted polynucleotide in a suitable cell host, this polynucleotide being amplified every time that the recombinant vector replicates. The inserted polynucleotide can be one that encodes SPANK polypeptides of the invention.

A second preferred embodiment of the recombinant vectors according to the invention, consists of expression vectors comprising polynucleotides encoding SPANK polypeptides of the invention. Within certain embodiments, expression vectors are employed to express a SPANK polypeptide of the invention, preferably a modified SPANK polypeptide or fragment described in the present invention, which can be then purified and, for example, be used to inhibit IRAP binding or as a treatment for insulin resistance.

Expression requires that appropriate signals are provided in the vectors, the signals including various regulatory elements, such as enhancers/promoters from both viral and mammalian sources, that drive expression of the SPANKs of interest in host cells. Dominant drug selection markers for establishing permanent, stable, cell clones expressing the products are generally included in the expression vectors of the invention, as they are elements that link expression of the drug selection markers to expression of the polypeptide.

More particularly, the present invention relates to expression vectors which include nucleic acids encoding a SPANK polypeptide of the invention, or a modified SPANK polypeptide as described herein, or variants or fragments thereof, under the control of a regulatory sequence for SPANK polypeptides, or alternatively under the control of an exogenous regulatory sequence.

Consequently, preferred expression vectors of the invention are selected from the group consisting of : (a) a SPANK polypeptide regulatory sequence that drives the expression of a coding polynucleotide operably linked thereto; and (b) a SPANK polypeptide coding sequence of the invention, operably linked to regulatory sequences allowing its expression in a suitable cell host and/or host organism.

Some of the elements which can be found in the vectors of the present invention are described in further detail in the following sections.

1) General features of the expression vectors of the invention:

A recombinant vector according to the invention comprises, but is not limited to, a YAC (Yeast Artificial Chromosome), a BAC (Bacterial Artificial Chromosome), a phage, a phagemid, a cosmid, a plasmid, or even a linear DNA molecule which may consist of a chromosomal, non-chromosomal, semi-synthetic or synthetic DNA. Such a recombinant vector can comprise a transcriptional unit comprising an assembly of :

(1) a genetic element or elements having a regulatory role in SPANK expression, for example promoters or enhancers. Enhancers are cis-acting elements of DNA, usually from about 10 to 300 bp in length that act on the promoter to increase the transcription.

(2) a structural or coding sequence which is transcribed into mRNA and eventually translated into a polypeptide, said structural or coding sequence being operably linked to the regulatory elements described in (1); and

(3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, when a recombinant protein is expressed without a leader or transport sequence, it may include a N-terminal residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

Generally, recombinant expression vectors will include origins of replication, selectable markers permitting transformation of the host cell, and a promoter derived from a highly expressed gene to direct transcription of a downstream structural sequence. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably a leader sequence capable of directing secretion of the translated protein into the periplasmic space or the extracellular medium. In a specific embodiment wherein the vector is adapted for transfecting and expressing desired sequences in mammalian host cells, preferred vectors will comprise an origin of replication in the desired host, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5'-flanking non-transcribed sequences. DNA sequences derived from the SV40 viral genome, for example SV40 origin, early promoter, enhancer, splice and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

2) Regulatory elements

Promoters

The suitable promoter regions used in the expression vectors according to the present invention are chosen taking into account the cell host in which the heterologous SPANK has to be expressed. The particular promoter employed to control the expression of a nucleic acid sequence of interest is not believed to be important, so long as it is capable of directing the expression of the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell, such as, for example, a human or a viral promoter.

A suitable promoter may be heterologous with respect to the nucleic acid for which it controls the expression or alternatively can be endogenous to the native polynucleotide containing the coding sequence to be expressed. Additionally, the promoter is generally heterologous with respect to the recombinant vector sequences within which the construct promoter/coding sequence has been inserted.

Promoter regions can be selected from any desired gene using, for example, CAT (chloramphenicol transferase) vectors and more preferably pKK232-8 and pCM7 vectors.

Preferred bacterial promoters are the LacI, LacZ, the T3 or T7 bacteriophage RNA polymerase promoters, the gpt, lambda PR, PL and trp promoters (EP 0036776), the polyhedrin promoter, or the p10 protein promoter from baculovirus (Kit Novagen) (Smith *et al.*, 1983; O'Reilly *et al.*, 1992), the lambda PR promoter or also the trc promoter.

Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-L. Selection of a convenient vector and promoter is well within the level of ordinary skill in the art.

The choice of a promoter is well within the ability of a person skilled in the field of genetic egineering. For example, one may refer to the book of Sambrook *et al.* (1989) or also to the procedures described by Fuller *et al.* (1996).

Other regulatory elements

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the SPANK transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed such as human growth hormone and SV40 polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

Vectors containing the appropriate DNA sequence as described above can be utilized to transform an appropriate host to allow the expression of the desired polypeptide or polynucleotide.

3) Selectable markers

Such markers would confer an identifiable change to the cell permitting easy identification of cells containing the expression construct. The selectable marker genes for selection of transformed host cells are preferably dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, TRP1 for S. cerevisiae or tetracycline, rifampicin or ampicillin resistance in *E. coli*, or levan saccharase for mycobacteria, this latter marker being a negative selection marker.

4) Vectors

Bacterial vectors

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and a bacterial origin of replication derived from commercially available plasmids comprising genetic

elements of pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia, Uppsala, Sweden), and GEM1 (Promega Biotec, Madison, WI, USA).

Large numbers of other suitable vectors are known to those of skill in the art, and are commercially available, such as the following bacterial vectors : pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); pWLNEO, pSV2CAT, pOG44, pXT1, pSG (Stratagene); pSVK3, pBPV, pMSG, pSVL (Pharmacia); pQE-30 (QIAexpress).

Baculovirus vectors

A suitable vector for the expression polypeptides of the invention is a baculovirus vector that can be propagated in insect cells and in insect cell lines. A specific suitable host vector system is the pVL1392/1393 baculovirus transfer vector (Pharmingen) that is used to transfect the SF9 cell line (ATCC N°CRL 1711) which is derived from *Spodoptera frugiperda*.

Other suitable vectors for the expression of a SPANK polypeptide in a baculovirus expression system include those described by Chai *et al.* (1993), Vlasak *et al.* (1983) and Lenhard *et al.* (1996).

Viral vectors

In one specific embodiment, the vector is derived from an adenovirus. Examples of adenovirus vectors that can be used are those described by Feldman and Steg (1996) or Ohno *et al.* (1994), or alternatively, the human adenovirus type 2 or 5 (Ad 2 or Ad 5) or an adenovirus of animal origin (French patent application N° FR-93.05954).

Retrovirus vectors and adeno-associated virus vectors can be used for the transfer of exogenous polynucleotides *in vivo*, particularly to mammals, including humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host.

Retroviruses useful for the preparation or construction of retroviral *in vitro* or *in vivo* SPANK delivery vehicles of the present invention include retroviruses selected from the group consisting of Mink-Cell Focus Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis virus and Rous Sarcoma virus. Useful Murine Leukemia Viruses include the 4070A and the 1504A viruses, Abelson (ATCC No VR-999), Friend (ATCC No VR-245), Gross (ATCC No VR-590), Rauscher (ATCC No VR-998) and Moloney Murine Leukemia Virus (ATCC No VR-190; PCT Application No WO 94/24298). Useful Rous Sarcoma Viruses include Bryan high titer (ATCC Nos VR-334, VR-657, VR-726, VR-659 and VR-728). Other useful retroviral vectors are those described in Roth *et al.* (1996), PCT Application No WO 93/25234, PCT Application No WO 94/ 06920, Roux *et al.*, 1989, Julan *et al.*, 1992 and Neda *et al.*, 1991.

Yet another viral vector system that is contemplated by the invention consists of the adeno-associated virus (AAV). The adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle (Muzyczka *et al.*, 1992). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (Flotte *et al.*, 1992; Samulski *et al.*, 1989; McLaughlin *et al.*, 1989). One advantageous feature of AAV derives from its reduced efficacy for transducing primary cells relative to transformed cells.

5) Delivery of the recombinant vectors

In order to effect expression of the polynucleotides of the invention, these constructs must be delivered into a cell. This delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cell lines, or *in vivo* or *ex vivo*, as in the treatment of certain disease states.

One mechanism is viral infection where the expression construct is encapsulated in an infectious viral particle.

Several non-viral methods for the transfer of polynucleotides into cultured mammalian cells are also contemplated by the present invention, and include, without being limited to, calcium phosphate precipitation (Graham *et al.*, 1973; Chen *et al.*, 1987;), DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland *et al.*, 1985), DNA-loaded liposomes (Nicolau *et al.*, 1982; Fraley *et al.*, 1979), and receptor-mediated transfection (Wu and Wu, 1987; 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

Once the expression polynucleotide has been delivered into the cell, it may be stably integrated into the genome of the recipient cell. This integration may be in the cognate location and orientation via homologous recombination (SPANK replacement) or it may be integrated in a random, non-specific location (SPANK augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle.

One specific embodiment for a method for delivering a protein or peptide to the interior of a cell of a vertebrate *in vivo* comprises the step of introducing a preparation comprising a physiologically acceptable carrier and a naked polynucleotide operatively coding for the polypeptide of interest into the interstitial space of a tissue comprising the cell, whereby the naked polynucleotide is taken up into the interior of the cell and has a physiological effect. This is particularly applicable for transfer *in vitro* but it may be applied to *in vivo* as well.

Compositions for use *in vitro* and *in vivo* comprising a "naked" polynucleotide are described in PCT application No. WO 90/11092 (Vical Inc.) and also in PCT application No. WO 95/11307 (Institut Pasteur, INSERM, Université d'Ottawa) as well as in the articles of Tacson *et al.* (1996) and of Huygen *et al.* (1996).

In still another embodiment of the invention, the transfer of a naked polynucleotide of the invention, including a polynucleotide construct of the invention, into cells may be proceeded with a particle bombardment (biolistic), said particles being DNA-coated microprojectiles accelerated to a high velocity allowing them to pierce cell membranes and enter cells without killing them, such as described by Klein *et al.* (1987).

In a further embodiment, the polynucleotide of the invention may be entrapped in a liposome (Ghosh and Bacchawat, 1991; Wong *et al.*, 1980; Nicolau *et al.*, 1987). These liposomes may further be targeted to cells expressing SPANK by methods well-known in the art.

In a specific embodiment, the invention provides a composition for the *in vivo* production of a SPANK polypeptide described herein. It comprises a naked polynucleotide operatively coding for this polypeptide, in solution in a physiologically acceptable carrier, and suitable for introduction into a tissue to cause cells of the tissue to express the said polypeptide.

The amount of vector to be injected to the desired host organism varies according to the site of injection. As an indicative dose, it will be injected between 0,1 and 100 μg of the vector in an animal body, preferably a mammal body, for example a mouse body.

In another embodiment of the vector according to the invention, it may be introduced in vitro in a host cell, preferably in a host cell previously harvested from the animal to be treated and more preferably a somatic cell such as a muscle cell. In a subsequent step, the cell that has been transformed with the vector coding for the desired SPANK polypeptide or the desired fragment thereof is reintroduced into the animal body in order to deliver the recombinant protein within the body either locally or systemically.

Other Polynucleotide Constructs

The terms "polynucleotide construct" and "recombinant polynucleotide" are used interchangeably herein to refer to linear or circular, purified or isolated polynucleotides that have been artificially designed and which comprise

at least two nucleotide sequences that are not found as contiguous nucleotide sequences in their initial natural environment.

SPANK Expression In Recombinant Cell Hosts And In Transgenic Animals.

In order to study the physiological and phenotypic consequences of a lack of synthesis of the SPANK protein, both at the cell level and at the multi cellular organism level, the invention also encompasses DNA constructs and recombinant vectors enabling a conditional expression of a specific allele of the SPANK cDNA and also of a copy of this cDNA harboring substitutions, deletions, or additions of one or more bases as regards to the SPANK nucleotide sequence of SEQ ID Nos 2, 3, or 5, or a fragment thereof.

The present invention embodies recombinant vectors comprising any one of the polynucleotides described in the present invention. A DNA construct can be based on the tetracycline resistance operon *tet* from *E. coli* transposon Tn10 for controlling SPANK expression, such as described by Gossen *et al.*(1992, 1995) and Furth *et al.*(1994). Such a DNA construct contains seven *tet* operator sequences from Tn10 (*tet*op) that are fused to either a minimal promoter or a 5'-regulatory sequence of SPANK, said minimal promoter or said SPANK regulatory sequence being operably linked to a polynucleotide of interest that codes either for a sense or an antisense oligonucleotide or for a polypeptide, including a SPANK polypeptide or a peptide fragment thereof. This DNA construct is functional as a conditional expression system for the nucleotide sequence of interest when the same cell also comprises a nucleotide sequence coding for either the wild type (tTA) or the mutant (rTA) repressor fused to the activating domain of viral protein VP16 of herpes simplex virus, placed under the control of a promoter, such as the HCMVIE1 enhancer/promoter or the MMTV-LTR. Indeed, a DNA construct of the invention comprises both the polynucleotide containing the *tet* operator sequences and the polynucleotide containing a sequence coding for the tTA or the rTA repressor.

In a specific embodiment, the conditional expression DNA construct contains the sequence encoding the mutant tetracycline repressor rTA, the expression of the polynucleotide of interest is silent in the absence of tetracycline and induced in its presence.

DNA Constructs Allowing Homologous Recombination: Replacement Vectors

Another DNA construct can comprise, from 5'-end to 3'-end: (a) a first nucleotide sequence that is comprised in the SPANK sequence; (b) a nucleotide sequence comprising a positive selection marker, such as the marker for neomycine resistance (*neo*); and (c) a second nucleotide sequence that is comprised in the SPANK sequence, and is located downstream of the first SPANK nucleotide sequence (a).

In a preferred embodiment, this DNA construct also comprises a negative selection marker located upstream the nucleotide sequence (a) or downstream the nucleotide sequence (c). Preferably, the negative selection marker comprises the thymidine kinase (*tk*) gene (Thomas *et al.*, 1986), the hygromycine beta gene (Te Riele *et al.*, 1990), the *hprt* gene (Van der Lugt *et al.*, 1991; Reid *et al.*, 1990) or the Diphteria toxin A fragment (*Dt-A*) gene (Nada *et al.*, 1993; Yagi *et al.*1990). These replacement vectors are described, for example, by Thomas *et al.*(1986; 1987), Mansour *et al.*(1988) and Koller *et al.*(1992).

The size of the nucleotide sequences (a) and (c) ranges from 1 to 6 kb or from 1 to 4 kb, or 1 to 2 kb.

DNA Constructs Allowing Homologous Recombination: Cre-LoxP System.

These new DNA constructs make use of the site specific recombination system of the P1 phage. The P1 phage possesses a recombinase called Cre that interacts specifically with a 34 base pairs *lox*P site. The *lox*P site is composed of two palindromic sequences of 13 bp separated by a 8 bp conserved sequence (Hoess *et al.*, 1986). The recombination by the Cre enzyme between two *lox*P sites having an identical orientation leads to the deletion of the DNA fragment.

The Cre-*lox*P system used in combination with a homologous recombination technique has been first described by Gu *et al.*(1993, 1994). Briefly, a nucleotide sequence of interest to be inserted in a targeted location of the genome

harbors at least two *lox*P sites in the same orientation and located at the respective ends of a nucleotide sequence to be excised from the recombinant genome. The excision event requires the presence of the recombinase (Cre) enzyme within the nucleus of the recombinant cell host. The recombinase enzyme may be brought at the desired time either by (a) incubating the recombinant cell hosts in a culture medium containing this enzyme, by injecting the Cre enzyme directly into the desired cell, such as described by Araki *et al.*(1995), or by lipofection of the enzyme into the cells, such as described by Baubonis *et al.*(1993); (b) transfecting the cell host with a vector comprising the *Cre* coding sequence operably linked to a promoter functional in the recombinant cell host, which promoter being optionally inducible, said vector being introduced in the recombinant cell host, such as described by Gu *et al.*(1993) and Sauer *et al.*(1988); (c) introducing in the genome of the cell host a polynucleotide comprising the *Cre* coding sequence operably linked to a promoter functional in the recombinant cell host, which promoter is optionally inducible, and said polynucleotide being inserted in the genome of the cell host either by a random insertion event or an homologous recombination event, such as described by Gu *et al.*(1994).

In a specific embodiment, the vector containing the sequence to be inserted in SPANK by homologous recombination is constructed in such a way that selectable markers are flanked by *lox*P sites of the same orientation. It is possible, by treatment by the Cre enzyme, to eliminate the selectable markers while leaving the SPANK sequences of interest that have been inserted by an homologous recombination event. Again, two selectable markers are needed: a positive selection marker to select for the recombination event and a negative selection marker to select for the homologous recombination event. Vectors and methods using the Cre-*lox*P system are described by Zou *et al.*(1994).

Thus, an additional DNA construct of the invention comprises, from 5'-end to 3'-end: (a) a first nucleotide sequence that is comprised in the SPANK sequence; (b) a nucleotide sequence comprising a polynucleotide encoding a positive selection marker, said nucleotide sequence comprising additionally two sequences defining a site recognized by a recombinase, such as a *lox*P site, the two sites being placed in the same orientation; and (c) a second nucleotide sequence that is comprised in the SPANK sequence, and is located on the genome downstream of the first SPANK nucleotide sequence (a).

The sequences defining a site recognized by a recombinase, such as a *lox*P site, are preferably located within the nucleotide sequence (b) at suitable locations bordering the nucleotide sequence for which the conditional excision is sought. In one specific embodiment, two *lox*P sites are located at each side of the positive selection marker sequence, in order to allow its excision at a desired time after the occurrence of the homologous recombination event. In a preferred embodiment of a method using the third DNA construct described above, the excision of the polynucleotide fragment bordered by the two sites recognized by a recombinase, preferably two loxP sites, is performed at a desired time, due to the presence within the genome of the recombinant host cell of a sequence encoding the Cre enzyme operably linked to a promoter sequence, preferably an inducible promoter, more preferably a tissue-specific promoter sequence and most preferably a promoter sequence which is both inducible and tissue-specific, such as described by Gu *et al.*(1994).

The presence of the Cre enzyme within the genome of the recombinant cell host may result from the breeding of two transgenic animals, the first transgenic animal bearing the SPANK-derived sequence of interest containing the *lox*P sites as described above and the second transgenic animal bearing the *Cre* coding sequence operably linked to a suitable promoter sequence, such as described by Gu *et al.*(1994).

Spatio-temporal control of the Cre enzyme expression may also be achieved with an adenovirus based vector that contains the Cre gene thus allowing infection of cells, or *in vivo* infection of organs, for delivery of the Cre enzyme, such as described by Anton and Graham (1995) and Kanegae *et al.*(1995).

The DNA constructs described above may be used to introduce a desired nucleotide sequence of the invention, preferably a SPANK cDNA sequence, or an altered copy of a SPANK cDNA sequence, within a

predetermined location of the targeted genome, leading either to the generation of an altered copy of a targeted SPANK (knock-out homologous recombination) or to the replacement of a copy of the targeted SPANK by another copy sufficiently homologous to allow an homologous recombination event to occur (knock-in homologous recombination).

Nuclear Antisense DNA Constructs.

Other compositions containing a vector of the invention comprising an oligonucleotide fragment of the nucleic sequence SEQ ID No 2, 3, or, 5, preferably a fragment including the start codon of SPANK, as an antisense tool that inhibits the expression of SPANK. Preferred methods using antisense polynucleotide according to the present invention are the procedures described by Sczakiel *et al.*(1995) or those described in PCT Application No WO 95/24223, the disclosures of which are incorporated by reference herein in their entirety.

Preferably, the antisense tools are chosen among the polynucleotides (15-200 bp long) that are complementary to the 5'end of the SPANK mRNA. In one embodiment, a combination of different antisense polynucleotides complementary to different parts of the desired targeted SPANK are used.

Preferred antisense polynucleotides according to the present invention are complementary to a sequence of the mRNAs of SPANK that contains the translation initiation codon ATG.

Preferably, the antisense polynucleotides of the invention have a 3' polyadenylation signal that has been replaced with a self-cleaving ribozyme sequence, such that RNA polymerase II transcripts are produced without poly(A) at their 3' ends, these antisense polynucleotides being incapable of export from the nucleus, such as described by Liu *et al.*(1994). In a preferred embodiment, these SPANK antisense polynucleotides also comprise, within the ribozyme cassette, a histone stem-loop structure to stabilize cleaved transcripts against 3'-5' exonucleolytic degradation, such as the structure described by Eckner *et al.*(1991).

Oligonucleotide Probes And Primers

Polynucleotides derived from SPANK are useful in order to detect the presence of at least a copy of a nucleotide sequence of SEQ ID No 2, 3, or 5, or a fragment, complement, or variant thereof in a test sample.

Particularly preferred probes and primers of the invention include isolated, purified, or recombinant polynucleotides comprising a contiguous span of at least 12, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500, or 1000 nucleotides of SEQ ID No 2, 3, 5 or the complements thereof.

IV. Recombinant Cells of the Invention

Another object of the invention consists of host cells that have been transformed or transfected with one of the polynucleotides described herein, and more precisely a polynucleotide comprising a polynucleotide encoding a SPANK polypeptide of the invention such as any one of those described in "Polynucleotides of the Invention". These polynucleotides can be present in cells as a result of transient or stable transfection. The invention includes host cells that are transformed (prokaryotic cells) or that are transfected (eukaryotic cells) with a recombinant vector such as any one of those described in « Recombinant Vectors of the Invention ».

Generally, a recombinant host cell of the invention comprises at least one of the polynucleotides or the recombinant vectors of the invention that are described herein.

Preferred host cells used as recipients for the recombinant vectors of the invention are the following :

a) Prokaryotic host cells : *Escherichia coli* strains (I.E. DH5-α strain), *Bacillus subtilis*, *Salmonella typhimurium*, and strains from species like *Pseudomonas*, *Streptomyces* and *Staphylococcus*, and

b) Eukaryotic host cells : HeLa cells (ATCC N°CCL2; N°CCL2.1; N°CCL2.2), Cv 1 cells (ATCC N°CCL70), COS cells (ATCC N°CRL1650; N°CRL1651), Sf-9 cells (ATCC N°CRL1711), C127 cells (ATCC N° CRL-1804), 3T3

(ATCC N° CRL-6361), CHO (ATCC N° CCL-61), human kidney 293 (ATCC N° 45504; N° CRL-1573), BHK (ECACC N° 84100501; N° 84111301), PLC cells, HepG2, and Hep3B;

c) other mammalian host cells.

SPANK expression in mammalian, and typically human, cells may be rendered defective, or alternatively it may be preceeded with the insertion of a regulatory sequence or with the replacement of the SPANK counterpart in the genome of an animal cell by a SPANK polynucleotide according to the invention. These genetic alterations may be generated by homologous recombination events using specific DNA constructs that have been previously described.

One kind of cell hosts that may be used are mammal zygotes, such as murine zygotes. For example, murine zygotes may undergo microinjection with a purified DNA molecule of interest, for example a purified DNA molecule that has previously been adjusted to a concentration range from 1 ng/ml -for BAC inserts- 3 ng/μl -for P1 bacteriophage inserts- in 10 mM Tris-HCl, pH 7.4, 250 μM EDTA containing 100 mM NaCl, 30 μM spermine, and 70 μM spermidine. When the DNA to be microinjected has a large size, polyamines and high salt concentrations can be used in order to avoid mechanical breakage of this DNA, as described by Schedl et al (1993b).

Any one of the polynucleotides of the invention, including the DNA constructs described herein, may be introduced in an embryonic stem (ES) cell line, preferably a mouse ES cell line. ES cell lines are derived from pluripotent, uncommitted cells of the inner cell mass of pre-implantation blastocysts. Preferred ES cell lines are the following: ES-E14TG2a (ATCC n° CRL-1821), ES-D3 (ATCC n° CRL1934 and n° CRL-11632), YS001 (ATCC n° CRL-11776), 36.5 (ATCC n° CRL-11116). To maintain ES cells in an uncommitted state, they are cultured in the presence of growth-inhibited feeder cells that provide the appropriate signals to preserve this embryonic phenotype and serve as a matrix for ES cell adherence. Preferred feeder cells are primary embryonic fibroblasts that are established from the tissue of day 13- day 14 embryos of virtually any mouse strain, that are maintained in culture, such as described by Abbondanzo *et al.*(1993) and are inhibited in growth by irradiation, such as described by Robertson (1987), or by the presence of an inhibitory concentration of LIF, such as described by Pease and Williams (1990).

The constructs in the host cells can be used in a conventional manner to produce the SPANK product encoded by the recombinant sequence.

Following transformation of a suitable host and growth of the host to an appropriate cell density, the selected promoter is induced by appropriate means, such as temperature shift or chemical induction, and cells are cultivated for an additional period.

Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in the expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known by the skilled artisan.

Further, according to the invention, these recombinant cells can be created *in vitro* or *in vivo* in an animal, preferably a mammal, most preferably selected from the group consisting of mice, rats, dogs, pigs, sheep, cattle, and primates, not to include humans. Recombinant cells created *in vitro* can also be later surgically implanted in an animal, for example. Methods to create recombinant cells *in vivo* in animals are well-known in the art.

The present invention also encompasses primary, secondary, and immortalized homologously recombinant host cells of vertebrate origin, preferably mammalian origin and particularly human origin, that have been engineered to: a) insert exogenous (heterologous) polynucleotides into the endogenous chromosomal DNA of a targeted gene, b) delete endogenous chromosomal DNA, and/or c) replace endogenous chromosomal DNA with exogenous polynucleotides. Insertions, deletions, and/or replacements of polynucleotide sequences may be to the coding

sequences of the targeted SPANK and/or to regulatory regions, such as promoter and enhancer sequences, operably associated with the targeted SPANK.

The present invention further relates to a method of making a homologously recombinant host cell in vitro or in vivo, wherein the expression of a targeted SPANK not normally expressed in the cell is altered. Preferably the alteration causes expression of the targeted SPANK under normal growth conditions or under conditions suitable for producing the polypeptide encoded by the targeted SPANK. The method comprises the steps of: (a) transfecting the cell in vitro or in vivo with a polynucleotide construct, the a polynucleotide construct comprising; (i) a targeting sequence; (ii) a regulatory sequence and/or a coding sequence; and (iii) an unpaired splice donor site, if necessary, thereby producing a transfected cell; and (b) maintaining the transfected cell in vitro or in vivo under conditions appropriate for homologous recombination.

The present invention further relates to a method of altering the expression of a targeted SPANK in a cell in vitro or in vivo wherein the SPANK is not normally expressed in the cell, comprising the steps of: (a) transfecting the cell in vitro or in vivo with a a polynucleotide construct, the a polynucleotide construct comprising: (i) a targeting sequence; (ii) a regulatory sequence and/or a coding sequence; and (iii) an unpaired splice donor site, if necessary, thereby producing a transfected cell; and (b) maintaining the transfected cell in vitro or in vivo under conditions appropriate for homologous recombination, thereby producing a homologously recombinant cell; and (c) maintaining the homologously recombinant cell in vitro or in vivo under conditions appropriate for expression of the gene.

The present invention further relates to a method of making a polypeptide of the present invention by altering the expression of a targeted endogenous SPANK in a cell in vitro or in vivo wherein the SPANK is not normally expressed in the cell, comprising the steps of: a) transfecting the cell in vitro with a a polynucleotide construct, the a polynucleotide construct comprising: (i) a targeting sequence; (ii) a regulatory sequence and/or a coding sequence; and (iii) an unpaired splice donor site, if necessary, thereby producing a transfected cell; (b) maintaining the transfected cell in vitro or in vivo under conditions appropriate for homologous recombination, thereby producing a homologously recombinant cell; and c) maintaining the homologously recombinant cell in vitro or in vivo under conditions appropriate for expression of the gene thereby making the polypeptide.

The present invention further relates to a a polynucleotide construct which alters the expression of a targeted SPANK in a cell type in which the SPANK is not normally expressed. This occurs when the polynucleotide construct is inserted into the chromosomal DNA of the target cell, wherein the a polynucleotide construct comprises: a) a targeting sequence; b) a regulatory sequence and/or coding sequence; and c) an unpaired splice-donor site, if necessary. Further included are polynucleotide constructs, as described above, wherein the construct further comprises a polynucleotide which encodes a polypeptide and is in-frame with the targeted endogenous SPANK after homologous recombination with chromosomal DNA.

The compositions may be produced, and methods performed, by techniques known in the art, such as those described in U.S. Patent Nos: 6,054,288; 6,048,729; 6,048,724; 6,048,524; 5,994,127; 5,968,502; 5,965,125; 5,869,239; 5,817,789; 5,783,385; 5,733,761; 5,641,670; 5,580,734 ; International Publication Nos:WO96/29411, WO 94/12650; and scientific articles including 1994; Koller *et al.*, Proc. Natl. Acad. Sci. USA 86:8932-8935 (1989) (the disclosures of each of which are incorporated by reference in their entireties).

V. Antibodies That Bind SPANK Polypeptides of the Invention

This invention encompasses antibodies, both polyclonal and monoclonal, that bind to SPANK or to a portion characteristic of or unique to SPANK.

Any SPANK polypeptide or whole protein may be used to generate antibodies capable of specifically binding to expressed SPANK protein or fragments thereof as described. The antibody compositions of the invention are

capable of specifically binding or specifically bind to the SPANK protein. For an antibody composition to specifically bind to SPANK, it must demonstrate at least a 5%, 10%, 15%, 20%, 25%, 50%, or 100% greater binding affinity for SPANK than for any other known protein in an ELISA, RIA, or other antibody-based binding assay.

In a preferred embodiment of the invention antibody compositions are capable of selectively binding, or selectively bind to an epitope-containing fragment of a polypeptide comprising a contiguous span of at least 3 amino acids, preferably at least 6 to 10 amino acids, more preferably at least 12, 15, 20, 25, 30, 40, 50, or 100 amino acids of SEQ ID NO: 1, SEQ ID NO: 4, or SEQ ID NO: 6, wherein said antibody composition is optionally either polyclonal or monoclonal.

The present invention also contemplates the use of polypeptides comprising a contiguous span of at least 3 amino acids, preferably at least 6 to 10 amino acids, more preferably at least 12, 15, 20, 25, 50, or 100 amino acids of a SPANK polypeptide in the manufacture of antibodies. In a preferred embodiment such polypeptides are useful in the manufacture of antibodies to detect the presence and absence of SPANK.

Non-human animals or mammals, whether wild-type or transgenic, which express a different species of SPANK than the one to which antibody binding is desired, and animals which do not express SPANK (*i.e.* a SPANK knock out animal as described in herein) are particularly useful for preparing antibodies. SPANK knock out animals will recognize all or most of the exposed regions of SPANK as foreign antigens, and therefore produce antibodies with a wider array of SPANK epitopes. Moreover, smaller polypeptides with only 10 to 30 amino acids may be useful in obtaining specific binding to particular fragments of SPANK, such as the ANK, SAM, or Parp regions as described above in Section I. In addition, the humoral immune system of animals which produce a species of SPANK that resembles the antigenic sequence will preferentially recognize the differences between the animal's native SPANK species and the antigen sequence, and produce antibodies to these unique sites in the antigen sequence. Such a technique will be particularly useful in obtaining antibodies that specifically bind to variants of SPANK.

Monoclonal or polyclonal antibody to the protein can then be prepared as follows:

Monoclonal Antibody Production by Hybridoma Fusion

Monoclonal antibody to epitopes in the SPANK protein or a portion thereof can be prepared from murine hybridomas according to the classical method of Kohler, G. and Milstein, C., Nature 256:495 (1975) or derivative methods thereof. Also see Harlow, E., and D. Lane. 1988. Antibodies A Laboratory Manual. Cold Spring Harbor Laboratory. pp. 53-242.

Briefly, a mouse is repetitively inoculated with a few micrograms of the SPANK protein or a portion thereof over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells, and the excess unfused cells destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted and aliquots of the dilution placed in wells of a microtiter plate where growth of the culture is continued. Antibody-producing clones are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures, such as ELISA, as originally described by Engvall, E., Meth. Enzymol. 70:419 (1980), and derivative methods thereof. Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. *et al.* Basic Methods in Molecular Biology Elsevier, New York. Section 21-2.

B. Polyclonal Antibody Production by Immunization

Polyclonal antiserum containing antibodies to heterogeneous epitopes in the SPANK protein or a portion thereof have been prepared by immunizing suitable non-human animal with the SPANK protein or a portion thereof, which can be unmodified or modified to enhance immunogenicity. A suitable non-human animal is preferably a non-human mammal is selected, usually a mouse, rat, rabbit, goat, or horse. Alternatively, a crude preparation that has

been enriched for SPANK concentration can be used to generate antibodies. Such proteins, fragments or preparations are introduced into the non-human mammal in the presence of an appropriate adjuvant (*e.g.* aluminum hydroxide, RIBI, etc.) which is known in the art. In addition the protein, fragment or preparation can be pretreated with an agent which will increase antigenicity, such agents are known in the art and include, for example, methylated bovine serum albumin (mBSA), bovine serum albumin (BSA), Hepatitis B surface antigen, and keyhole limpet hemocyanin (KLH). Serum from the immunized animal is collected, treated and tested according to known procedures. If the serum contains polyclonal antibodies to undesired epitopes, the polyclonal antibodies can be purified by immunoaffinity chromatography.

Effective polyclonal antibody production is affected by many factors related both to the antigen and the host species. Also, host animals vary in response to site of inoculations and dose, with both inadequate or excessive doses of antigen resulting in low titer antisera. Small doses (ng level) of antigen administered at multiple intradermal sites appears to be most reliable. Techniques for producing and processing polyclonal antisera are known in the art, see for example, Mayer and Walker (1987). An effective immunization protocol for rabbits can be found in Vaitukaitis, J. *et al.* J. Clin. Endocrinol. Metab. 33:988-991 (1971).

Booster injections can be given at regular intervals, and antiserum harvested when antibody titer thereof, as determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen, begins to fall. See, for example, Ouchterlony, O. *et al.*, Chap. 19 in: Handbook of Experimental Immunology D. Wier (ed) Blackwell (1973). Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum. Affinity of the antisera for the antigen is determined by preparing competitive binding curves, as described, for example, by Fisher, D., Chap. 42 in: Manual of Clinical Immunology, 2d Ed. (Rose and Friedman, Eds.) Amer. Soc. For Microbiol., Washington, D.C. (1980).

Antibody preparations prepared according to either the monoclonal or the polyclonal protocol are useful in quantitative immunoassays which determine concentrations of antigen-bearing substances in biological samples; they are also used semi-quantitatively or qualitatively to identify the presence of antigen in a biological sample. The antibodies may also be used in therapeutic compositions for killing cells expressing the protein or reducing the levels of the protein in the body.

The antibodies of the invention may be labeled by any one of the radioactive, fluorescent or enzymatic labels known in the art.

Consequently, the invention is also directed to a method for specifically detecting the presence of a SPANK polypeptide in a biological sample, said method comprising the following steps :

a) bringing into contact the biological sample with a polyclonal or monoclonal antibody that specifically binds a SPANK polypeptide, or to a peptide fragment or variant thereof; and

b) detecting the antigen-antibody complex formed.

The invention also concerns a diagnostic kit for detecting *in vitro* the presence of a SPANK polypeptide according to the present invention in a biological sample, wherein said kit comprises:

a) a polyclonal or monoclonal antibody that specifically binds a SPANK polypeptide, or to a peptide fragment or variant thereof, optionally labeled;

b) a reagent allowing the detection of the antigen-antibody complexes formed, said reagent carrying optionally a label, or being able to be recognized itself by a labeled reagent, more particularly in the case when the above-mentioned monoclonal or polyclonal antibody is not labeled by itself.

VI. Assays for Identifying Modulators of SPANK Activity

The work described herein supports a role for SPANK in regulating the sequestration of GLUT4 vesicles in cells and, thus, for SPANK's role as the distal effector in the insulin signaling pathway. In fact, SPANK is the only known insulin-signaling component that physically interacts with a constituent(s) of GLUT4 vesicles. Because it is a distal effector of insulin signaling – and apparently the most distal effector with respect to GLUT4 translocation – SPANK is of particular interest as a target for drugs to treat or prevent insulin resistance or diabetes. SPANK can be a target for hypoglycemic agents (*e.g.*, existing compounds or molecules; compounds or molecules designed/produced to act on SPANK) that act, for example, by displacing GLUT4/IRAP from SPANK by competitive binding or modulating the poly(ADP) ribose polymerase activity of SPANK, or by displacing SPANK from myosin.

Methods of screening for/identifying molecules or compounds (drugs) useful for altering or modulating GLUT4 translocation, glucose transport and/or insulin responsiveness are described herein and in the Examples 1-5 and 7-9. The present invention also provides methods for screening for/identifying drugs (molecules, compounds) useful for therapy/treatment of diabetes and insulin resistance as well as obesity-related diseases and disorders, muscle-related disorders, and general feelings of malaise. For example, the phosphorylation of SPANK or the PARP activity of SPANK, or IRAP binding or myosin binding can be used to screen for such drugs.

The insulin-stimulated phosphorylation of SPANK can be monitored based on the resultant gel mobility shift, *i.e.*, the approximately 10 kD increase in the apparent molecular weight of SPANK. For example, a cell culture can be treated with a compound of interest in the presence or absence of insulin. Cell lysates are then resolved by electrophoresis and immunoblotted with an anti-SPANK antibody. The effect of a compound on SPANK phosphorylation can be inferred from the characteristic gel mobility shift of SPANK.

The effect of a compound of interest on the PARP activity of SPANK can be assayed *in vivo* and *in vitro*. For example, cells can be pre-treated with the compound and cell lysates resolved by electrophoresis. PARP product (*i.e.*, poly(ADP-ribose)) can then be immunoblotted using commercial antibodies against poly(ADP-ribose) modifications. Alternatively, SPANK protein (either native or recombinant) can be purified and incubated *in vitro* with a substrate protein in the presence of the compound. *In vitro* PARP reaction can be quantitated using radiolabeled NAD as a PARP cofactor, as poly(ADP-ribosyl)ation results in the incorporation of the radiolabel into substrate proteins.

The effect of a compound of interest on the IRAP binding activity of SPANK can be assayed *in vitro* and *in vivo*.

The effect of a compound of interest on the myosin binding activity of SPANK can be assayed *in vitro* and *in vivo*.

Thus, the invention features methods of identifying one or more compounds that modulate SPANK activity *in vitro* or in cells, that includes providing potential compounds to be tested to the cells (or in the presence of SPANK *in vitro*), and where modulation of a SPANK activity identifies the useful one or more compounds. Exemplary assays of SPANK activity that may be used to identify modulators of SPANK activity are described in the Examples 1-5 and 7-9. To these assays would be added compounds to be tested for their inhibitory or stimulatory effect on SPANK activity. Other assays in which an effect on SPANK activity is observed based on the addition of test compounds can also be used to screen for modulators of SPANK activity. The essential step is to apply an unknown compound and then to monitor an assay for a change from what is seen in the absence of the unknown compound. A change is defined as something that is significantly different in the presence of the compound compared to the absence of the compound. In this case, significantly different would be an "increase" or a "decrease" in a measurable effect of at least 25%, at least 50%, at least 75%, at least 100%, or more than 100%.

Although not wishing to be limited to a particular theory, the inventors postulate four mechanisms by which SPANK may regulate GLUT4 targeting. First (Figure 2A), SPANK may act to sequester GLUT4 vesicles in the basal

state by tethering GLUT4 vesicles to myosin or other cytoskeletal elements. Upon insulin stimulation, SPANK undergoes phosphorylation, and the SPANK-dependent connectivity between GLUT4 and myosin is disrupted. This relieves the sequestration and allows GLUT4 vesicles to undergo exocytosis. In this model, insulin sensitivity can be improved by compounds that impair (such as through competition) SPANK binding to GLUT4 vesicles or to myosin.

In the second model (Figure 2B), SPANK allows GLUT4 vesicles to respond to insulin signaling by juxtaposing GLUT4 vesicles next to other signaling molecules known to regulate GLUT4 targeting. In support of this scaffolding model, SPANK apparently binds to an inositol kinase and an phosphoinositol phosphatase, which have been shown to enhance and suppress GLUT4 translocation, respectively.

In the third model (Figure 2C), the PARP activity of SPANK may respond to insulin signaling by poly(ADP-ribosyl)ating GLUT4 vesicles or their targeting machinery, which in turn causes GLUT4 translocation. This model is supported by the effect of niacinamide--a PARP inhibitor--in suppressing GLUT4 translocation *in vivo*. In this model, insulin sensitivity can be improved by compounds that either enhance PARP activity of SPANK, or suppress the glycohydrolase activity that reverses PARP reaction.

Lastly (Figure 2D), SPANK uses myosin as a motor to propel GLUT4 toward cell surface in response to insulin stimulation. In this model, compounds that enhance SPANK binding to GLUT4 vesicles and myosin may improve insulin sensitivity.

VII. Modulators of SPANK Activity

The invention further relates to drugs useful for altering or modulating GLUT4 transport, glucose transport and/or insulin responsiveness. The present invention provides a specific target for use in therapy/treatment of insulin resistance/diabetes. It is known that antidiabetic drugs (therapies/treatments of diabetes) cause side effects, due to a large extent to the diverse effects of insulin in the body. By targeting more distal, GLUT4- specific components of the signaling pathway, many of these adverse side effects can be avoided. SPANK provides a particularly useful target because it is a distal effector – and perhaps the most distal effector – and is in direct contact with GLUT4 vesicles. Several glucose-lowering strategies are now possible. For example, molecules or compounds that displace GLUT4 vesicles by saturating SPANK can be used as drugs to facilitate GLUT4 translocation and, thus, enhance glucose clearance. Small molecules, such as short peptides (*e.g.*, as few as six amino acids, since a six amino acid-stretch within IRAP's cytosolic domain is sufficient to bind GLUT4) or small organic molecules can be used to saturate SPANK. Such drugs can be identified in an assay in which candidate drugs compete with or displace SPANK from its interaction (binding) with GLUT4 vesicles or can be designed based on/with reference to knowledge of the SPANK sequence and structure. Alternatively a SPANK variant that, for example, binds GLUT4 but does not tether it (and, thus, permits its translocation or exocytosis) can be identified or designed. Alternatively, molecules or compounds that displace SPANK from myosin can be used to cause GLUT4 translocation. Insulin sensitivity may also be increased through the use of enhancers of the PARP activity of SPANK or through the use of a compound that protects PARP from hydrolysis by glycohydrolase (Aoki et al (1993) Biochimica et Biophysica Acta 1158:251-256).

Methods for screening substances interacting with a SPANK polypeptide

For the purpose of the present invention, a ligand means a molecule, such as a protein, a peptide, an antibody or any synthetic chemical compound capable of binding to SPANK protein or one of its fragments or variants or to modulate the expression of the polynucleotide coding for SPANK or a fragment or variant thereof.

In the ligand screening method according to the present invention, a biological sample or a defined molecule to be tested as a putative ligand of the SPANK protein is brought into contact with the corresponding purified SPANK protein, for example the corresponding purified recombinant SPANK protein produced by a recombinant cell host as described hereinbefore, in order to form a complex between this protein and the putative ligand molecule to be tested.

As an illustrative example, to study the interaction of the SPANK protein, or a fragment thereof as described herein, can be with drugs or small molecules, such as molecules generated through combinatorial chemistry approaches, the microdialysis coupled to HPLC method described by Wang *et al.* (1997) or the affinity capillary electrophoresis method described by Bush *et al.* (1997), the disclosures of which are incorporated by reference, can be used.

In further methods, peptides, drugs, fatty acids, lipoproteins, or small molecules which interact with the SPANK protein, or a fragment thereof may be identified using assays such as the following. The molecule to be tested for binding is labeled with a detectable label, such as a fluorescent, radioactive, or enzymatic tag and placed in contact with immobilized SPANK protein, or a fragment thereof under conditions which permit specific binding to occur. After removal of non-specifically bound molecules, bound molecules are detected using appropriate means.

Another object of the present invention comprises methods and kits for the screening of candidate substances that interact with SPANK polypeptide.

The present invention pertains to methods for screening substances of interest that interact with a SPANK protein or one fragment or variant thereof. By their capacity to bind covalently or non-covalently to a SPANK protein or to a fragment or variant thereof, these substances or molecules may be advantageously used both *in vitro* and *in vivo*.

In vitro, said interacting molecules may be used as detection means in order to identify the presence of a SPANK protein in a sample, preferably a biological sample.

A method for the screening of a candidate substance comprises:

a) providing a polypeptide comprising, consisting essentially of, or consisting of a SPANK protein or a fragment as described previously;

b) obtaining a candidate substance;

c) bringing into contact said polypeptide with said candidate substance;

d) detecting the complexes formed between said polypeptide and said candidate substance.

The invention further concerns a kit for the screening of a candidate substance interacting with the SPANK polypeptide, wherein said kit comprises :

a) a SPANK protein;

b) optionally means useful to detect the complex formed between the SPANK protein or a peptide fragment or a variant thereof and the candidate substance.

In a preferred embodiment of the kit described above, the detection means comprises a monoclonal or polyclonal antibodies directed against the SPANK protein or a peptide fragment or a variant thereof.

Various candidate substances or molecules can be assayed for interaction with a SPANK polypeptide. These substances or molecules include, without being limited to, natural or synthetic organic compounds or molecules of biological origin such as polypeptides. When the candidate substance or molecule comprises a polypeptide, this polypeptide may be the resulting expression product of a phage clone belonging to a phage-based random peptide library, or alternatively the polypeptide may be the resulting expression product of a cDNA library cloned in a vector suitable for performing a two-hybrid screening assay.

The invention also pertains to kits useful for performing the hereinbefore described screening method. Preferably, such kits comprise a SPANK polypeptide or a fragment or a variant thereof, and optionally means useful to detect the complex formed between the SPANK polypeptide or its fragment or variant and the candidate substance. In a preferred embodiment the detection means comprise a monoclonal or polyclonal antibodies directed against the corresponding SPANK polypeptide or a fragment or a variant thereof.

A. Candidate ligands obtained from random peptide libraries

In a particular embodiment of the screening method, the putative ligand is the expression product of a DNA insert contained in a phage vector (Parmley and Smith, 1988). Specifically, random peptide phages libraries are used. The random DNA inserts encode for peptides of 8 to 20 amino acids in length (Oldenburg K.R. *et al.*, 1992; Valadon P., *et al.*, 1996; Lucas A.H., 1994; Westerink M.A.J., 1995; Felici F. *et al.*, 1991). According to this particular embodiment, the recombinant phages expressing a protein that binds to the immobilized SPANK protein is retained and the complex formed between the SPANK protein and the recombinant phage may be subsequently immunoprecipitated by a polyclonal or a monoclonal antibody directed against the SPANK protein.

Once the ligand library has been constructed in recombinant phage, the phage population is brought into contact with the immobilized SPANK protein. Then the preparation containing complexes is washed in order to remove the non-specifically bound recombinant phages. The phages that bind specifically to the SPANK protein are then eluted by a buffer (acid pH) or immunoprecipitated by the monoclonal antibody produced by the hybridoma anti-SPANK, and this phage population is subsequently amplified by an over-infection of bacteria (for example *E. coli*). The selection step may be repeated several times, preferably 2-4 times, in order to select the more specific recombinant phage clones. The last step comprises characterizing the peptide produced by the selected recombinant phage clones either by expression in infected bacteria and isolation, expressing the phage insert in another host-vector system, or sequencing the insert contained in the selected recombinant phages.

B. Candidate ligands obtained by competition experiments

Alternatively, peptides, drugs or small molecules which bind to the SPANK protein, or a fragment thereof, may be identified in competition experiments. In such assays, the SPANK protein, or a fragment thereof, is immobilized to a surface, such as a plastic plate. Increasing amounts of the peptides, drugs or small molecules are placed in contact with the immobilized SPANK protein, or a fragment thereof, in the presence of a detectable labeled known SPANK protein ligand. For example, the SPANK ligand may be detectably labeled with a fluorescent, radioactive, or enzymatic tag. The ability of the test molecule to bind the SPANK protein, or a fragment thereof, is determined by measuring the amount of detectably labeled known ligand bound in the presence of the test molecule. A decrease in the amount of known ligand bound to the SPANK protein, or a fragment thereof, when the test molecule is present indicated that the test molecule is able to bind to the SPANK protein, or a fragment thereof.

C. Candidate ligands obtained by affinity chromatography.

Proteins or other molecules interacting with the SPANK protein, or a fragment thereof, can also be found using affinity columns which contain the **SPANK** protein, or a fragment thereof. The SPANK protein, or a fragment thereof, may be attached to the column using conventional techniques including chemical coupling to a suitable column matrix such as agarose, Affi Gel®, or other matrices familiar to those of skill in art. In some embodiments of this method, the affinity column contains chimeric proteins in which the SPANK protein, or a fragment thereof, is fused to glutathion S transferase (GST). A mixture of cellular proteins or pool of expressed proteins as described above is applied to the affinity column. Proteins or other molecules interacting with the SPANK protein, or a fragment thereof, attached to the column can then be isolated and analyzed on 2-D electrophoresis gel as described in Ramunsen *et al.* (1997), the disclosure of which is incorporated by reference. Alternatively, the proteins retained on the affinity column can be purified by electrophoresis based methods and sequenced. The same method can be used to isolate antibodies, to screen phage display products, or to screen phage display human antibodies.

D. Candidate ligands obtained by optical biosensor methods

Proteins interacting with the SPANK protein, or a fragment thereof, can also be screened by using an Optical Biosensor as described in Edwards and Leatherbarrow (1997) and also in Szabo *et al.* (1995), the disclosure of which is incorporated by reference. This technique permits the detection of interactions between molecules in real time,

without the need of labeled molecules. This technique is based on the surface plasmon resonance (SPR) phenomenon. Briefly, the candidate ligand molecule to be tested is attached to a surface (such as a carboxymethyl dextran matrix). A light beam is directed towards the side of the surface that does not contain the sample to be tested and is reflected by said surface. The SPR phenomenon causes a decrease in the intensity of the reflected light with a specific association of angle and wavelength. The binding of candidate ligand molecules cause a change in the refraction index on the surface, which change is detected as a change in the SPR signal. For screening of candidate ligand molecules or substances that are able to interact with the SPANK protein, or a fragment thereof, the SPANK protein, or a fragment thereof, is immobilized onto a surface. This surface comprises one side of a cell through which flows the candidate molecule to be assayed. The binding of the candidate molecule on the SPANK protein, or a fragment thereof, is detected as a change of the SPR signal. The candidate molecules tested may be proteins, peptides, carbohydrates, lipids, or small molecules generated by combinatorial chemistry. This technique may also be performed by immobilizing eukaryotic or prokaryotic cells or lipid vesicles exhibiting an endogenous or a recombinantly expressed SPANK protein at their surface.

The main advantage of the method is that it allows the determination of the association rate between the SPANK protein and molecules interacting with the SPANK protein. It is thus possible to select specifically ligand molecules interacting with the SPANK protein, or a fragment thereof, through strong or conversely weak association constants.

E. Candidate ligands obtained through a two-hybrid screening assay.

The yeast two-hybrid system is designed to study protein-protein interactions *in vivo* (Fields and Song, 1989), and relies upon the fusion of a bait protein to the DNA binding domain of the yeast Gal4 protein. This technique is also described in the US Patent N° US 5,667,973 and the US Patent N° 5,283,173 (Fields *et al.*) the technical teachings of both patents being herein incorporated by reference.

The general procedure of library screening by the two-hybrid assay may be performed as described by Harper *et al.* (1993) or as described by Cho *et al.* (1998) or also Fromont-Racine *et al.* (1997).

The bait protein or polypeptide comprises, consists essentially of, or consists of a SPANK polypeptide or a fragment thereof.

More precisely, the nucleotide sequence encoding the SPANK polypeptide or a fragment or variant thereof is fused to a polynucleotide encoding the DNA binding domain of the GAL4 protein, the fused nucleotide sequence being inserted in a suitable expression vector, for example pAS2 or pM3.

Then, a human cDNA library is constructed in a specially designed vector, such that the human cDNA insert is fused to a nucleotide sequence in the vector that encodes the transcriptional domain of the GAL4 protein. Preferably, the vector used is the pACT vector. The polypeptides encoded by the nucleotide inserts of the human cDNA library are termed "prey" polypeptides.

A third vector contains a detectable marker gene, such as beta galactosidase gene or CAT gene that is placed under the control of a regulation sequence that is responsive to the binding of a complete Gal4 protein containing both the transcriptional activation domain and the DNA binding domain. For example, the vector pG5EC may be used.

Two different yeast strains are also used. As an illustrative but non limiting example the two different yeast strains may be the followings :

- Y190, the phenotype of which is (*MATa, Leu2-3, 112 ura3-12, trp1-901, his3-D200, ade2-101, gal4Dgal180D URA3 GAL-LacZ, LYS GAL-HIS3, cyh*^{r});
- Y187, the phenotype of which is (*MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3, -112 URA3 GAL-lacZmet*), which is the opposite mating type of Y190.

Briefly, 20 μg of pAS2/ SPANK and 20 μg of pACT-cDNA library are co-transformed into yeast strain Y190. The transformants are selected for growth on minimal media lacking histidine, leucine and tryptophan, but containing the histidine synthesis inhibitor 3-AT (50 mM). Positive colonies are screened for beta galactosidase by filter lift assay. The double positive colonies (*His*⁺, *beta-gal*⁺) are then grown on plates lacking histidine, leucine, but containing tryptophan and cycloheximide (10 mg/ml) to select for loss of pAS2/ SPANK plasmids bu retention of pACT-cDNA library plasmids. The resulting Y190 strains are mated with Y187 strains expressing SPANK or non-related control proteins; such as cyclophilin B, lamin, or SNF1, as *Gal4* fusions as described by Harper *et al.* (1993) and by Bram *et al.* (Bram RJ *et al.*, 1993), and screened for beta galactosidase by filter lift assay. Yeast clones that are *beta gal-* after mating with the control *Gal4* fusions are considered false positives.

In another embodiment of the two-hybrid method according to the invention, interaction between the SPANK or a fragment or variant thereof with cellular proteins may be assessed using the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech). As described in the manual accompanying the Matchmaker Two Hybrid System 2 (Catalog No. K1604-1, Clontech), the disclosure of which is incorporated herein by reference, nucleic acids encoding the **SPANK** protein or a portion thereof, are inserted into an expression vector such that they are in frame with DNA encoding the DNA binding domain of the yeast transcriptional activator GAL4. A desired cDNA, preferably human cDNA, is inserted into a second expression vector such that they are in frame with DNA encoding the activation domain of GAL4. The two expression plasmids are transformed into yeast and the yeast are plated on selection medium which selects for expression of selectable markers on each of the expression vectors as well as GAL4 dependent expression of the HIS3 gene. Transformants capable of growing on medium lacking histidine are screened for GAL4 dependent lacZ expression. Those cells which are positive in both the histidine selection and the lacZ assay contain interaction between SPANK and the protein or peptide encoded by the initially selected cDNA insert.

VIII. Pharmaceutical Compositions of the Invention

The SPANK polypeptides of the invention, antibodies of the invention, and modulators of SPANK activity can be administered to a mammal, including a human patient, alone or in pharmaceutical compositions where they are mixed with suitable carriers or excipient(s). The pharmaceutical composition is then provided at a therapeutically effective dose. A therapeutically effective dose refers to that amount of SPANK polypeptides, antibodies, or modulators of SPANK activity sufficient to result in amelioration of symptoms of diseases or disorders as determined by the methods described herein. A therapeutically effective dosage of a SPANK polypeptide of the invention, SPANK antibody of the invention, or modulator of SPANK activity is that dosage that is adequate to ameliorate symptoms of diseases or disorders with continued periodic use or administration. Diseases or disorders that SPANK polypeptides, antibodies, or modulators could be used to treat or prevent include, but are not limited to, insulin resistance, glucose sensitivity, and disorders associated with type II diabetes. Techniques for formulation and administration of SPANK may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

Routes of Administration.

Suitable routes of administration include oral, rectal, transmucosal, or intestinal administration, parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal or intraocular injections. A particularly useful method of administering compounds involves surgical implantation, for example into the abdominal cavity of the recipient, of a device for delivering SPANK polypeptides, antibodies, or modulators over an extended period of time. Sustained release formulations of the invented medicaments particularly are contemplated.

Composition/Formulation

Pharmaceutical compositions and medicaments for use in accordance with the present invention may be formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries. Proper formulation is dependent upon the route of administration chosen.

Certain of the medicaments described herein will include a pharmaceutically acceptable carrier and at least one polypeptide that is a SPANK polypeptide of the invention, or a SPANK antibody of the invention, or a SPANK modulator of the invention. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer such as a phosphate or bicarbonate buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

Pharmaceutical preparations that can be taken orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with fillers such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable gaseous propellant, *e.g.,* carbon dioxide. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.,* gelatin, for use in an inhaler or insufflator, may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.,* by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.,* in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Aqueous suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder or lyophilized form for constitution with a suitable vehicle, such as sterile pyrogen-free water, before use.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained release materials have

been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days.

Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Effective Dosage.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve their intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes or encompasses a concentration point or range shown to decrease IRAP binding, for example in an *in vitro* system. Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50, (the dose lethal to 50% of the test population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds that exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50, with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. *(See, e.g.,* Fingl *et al.*, 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active compound which are sufficient to maintain the desired effects. Dosages necessary to achieve these effects will depend on individual characteristics and route of administration.

Dosage intervals can also be determined using the value for the minimum effective concentration. Compounds should be administered using a regimen that maintains plasma levels above the minimum effective concentration for 10-90% of the time, preferably between 30-90%; and most preferably between 50-90%. In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

A dosage range for the amount of a SPANK polypeptide of the invention, that can be administered on a daily or regular basis to achieve desired results, ranges from 0.1 - 50 mg/kg body mass, alternatively from 0.2 - 25 mg/kg,

or from 1.0 - 20 mg/kg, or from 2.0 - 10 mg/kg. Of course, these daily dosages can be delivered or administered in small amounts periodically during the course of a day.

IX. Methods of Modulating GLUT4 Translocation

In Cells

Also the subject of this invention is a method of modulating, such as increasing or decreasing, GLUT4 translocation in cells. Methods of altering or modulating (enhancing or reducing) GLUT4 translocation in cells are described herein and in the Examples 1-5 and 6-9 The terms "modulate", "increase", and "decrease" are used as previously defined. "Enhancing" or "reducing" are taken mean the same as "increasing" and "decreasing", respectively.

In one embodiment, the method comprises enhancing GLUT4 translocation in cells. This embodiment comprises contacting cells with a drug, under conditions that permit entry of the drug into the cells, wherein the drug acts upon SPANK protein and alters SPANK binding of GLUT4, resulting in GLUT4 translocation in the cell (movement from the interior of the cell to the cell surface, where it is available for enhancing glucose uptake). In this embodiment, the term "alters" includes interrupting interactions or binding of SPANK with GLUT4 or enhancing interactions or binding of SPANK with either GLUT4 or myosin.

In another embodiment in which GLUT4 translocation is enhanced, the method comprises contacting cells with a drug, under conditions that permit entry of the drug into the cells (under conditions appropriate for entry of the drug into the cell). The drug is a compound or molecule that acts upon SPANK protein and prevents SPANK from binding GLUT4. As a result, SPANK binding of GLUT4 is interrupted and GLUT4 undergoes translocation in the cell. The phrase "SPANK binding of GLUT4 is interrupted" includes preventing SPANK from binding to GLUT4 and disrupting existing SPANK-GLUT4 binding and also includes direct SPANK-GLUT4 binding and indirect binding of SPANK to GLUT4 through an adapter, such as IRAP. Alternatively, if SPANK acts by using myosin to translocate GLUT4 vesicles, then drugs that enhance interactions of SPANK with either GLUT4 or myosin can be used to promote GLUT4 translocation.

In Individuals

The invention is also drawn to methods of altering or modulating (enhancing or reducing) glucose transport and/or insulin responsiveness in individuals. Altering GLUT4 translocation in cells (to enhance or reduce translocation) can be carried out in an individual by administering to the individual a drug that acts upon SPANK and modulates GLUT4 translocation in cells, such as in muscle cells and adipocytes, with the result that glucose uptake by the cells is also enhanced or reduced.

A further subject of this invention is a method of enhancing glucose uptake in cells in an individual (*e.g.*, a human in need of therapy or treatment for insulin resistance or diabetes). In one embodiment, the method comprises administering to the individual a drug that alters SPANK binding of GLUT4 in cells of the individual, whereby GLUT4 undergoes translocation in the cell and is available for glucose uptake, whereby glucose uptake is enhanced in the individual. In another embodiment, the method comprises administering to the individual a drug which prevents SPANK binding of GLUT4 in cells of the individuals, with the result that SPANK binding of GLUT4 is interrupted, GLUT4 undergoes translocation in the cells and is available for glucose uptake and glucose uptake is enhanced in the individual.

In the embodiments in which an individual is treated, the drug is administered using known methods, such as by intramuscular injection, intravenous injection or oral administration. Drugs administered by these methods can be provided in a variety of forms and will usually be present in a physiologically compatible carrier (*e.g.*, saline, buffer). For example, such drugs can be administered in solutions appropriate for injection or infusion or for oral administration.

The amount or dose or a drug can be determined empirically and the amount or dose will vary depending on characteristics of the recipient such as age, gender, body size, severity of the condition (*e.g.*, insulin resistance or diabetes) and general health.

X. Methods of Treating Insulin-related Diseases and Disorders

A method of preventing or treating insulin-related diseases and disorders comprising providing a patient in need of such treatment with a SPANK polypeptide of the invention, SPANK antibody of the invention, or SPANK modulator of the invention. Preferably the SPANK polypeptide, antibody, or modulator is provided to the patient in a pharmaceutical composition that is preferably taken orally. Preferably the patient is a mammal, and most preferably a human. In preferred embodiments, the insulin-related disease or disorder is selected from the group consisting of insulin resistance, microangiopathic lesions resulting from Type II diabetes, ocular lesions caused by microangiopathy in individuals with Type II diabetes, and renal lesions caused by microangiopathy in individuals with Type II diabetes.

Other diseases or disorders that can be treated with SPANK polypeptides of the invention, or SPANK modulators of the invention, include obesity related diseases or disorders as well as muscle-related diseases and disorders and even general feelings of malaise. Muscle related diseases and disorders can be those associated with muscle fatigue, muscle atrophy, muscular dystrophy, injury through accident or surgery, or muscle building.

Methods For Inhibiting The Expression Of A SPANK Gene

Other therapeutic compositions according to the present invention comprise advantageously an oligonucleotide fragment of the nucleic sequence of SPANK as an antisense tool or a triple helix tool that inhibits the expression of the corresponding SPANK gene.

Antisense Approach

Preferred methods using antisense polynucleotide according to the present invention are the procedures described by Sczakiel *et al.*(1995).

Preferably, the antisense tools are chosen among the polynucleotides (15-200 bp long) that are complementary to the 5'end of the SPANK mRNA. In another embodiment, a combination of different antisense polynucleotides complementary to different parts of the desired targeted gene are used.

Preferred antisense polynucleotides according to the present invention are complementary to a sequence of the mRNAs of SPANK that contains the translation initiation codon ATG.

The antisense nucleic acids should have a length and melting temperature sufficient to permit formation of an intracellular duplex having sufficient stability to inhibit the expression of the SPANK mRNA in the duplex. Strategies for designing antisense nucleic acids suitable for use in gene therapy are disclosed in Green *et al.*, (1986) and Izant and Weintraub, (1984), the disclosures of which are incorporated herein by reference.

In some strategies, antisense molecules are obtained by reversing the orientation of the SPANK coding region with respect to a promoter so as to transcribe the opposite strand from that which is normally transcribed in the cell. The antisense molecules may be transcribed using in vitro transcription systems such as those which employ T7 or SP6 polymerase to generate the transcript. Another approach involves transcription of SPANK antisense nucleic acids in vivo by operably linking DNA containing the antisense sequence to a promoter in a suitable expression vector.

Alternatively, suitable antisense strategies are those described by Rossi *et al.*(1991), in the International Applications Nos. WO 94/23026, WO 95/04141, WO 92/18522 and in the European Patent Application No. EP 0 572 287 A2

An alternative to the antisense technology that is used according to the present invention comprises using ribozymes that will bind to a target sequence via their complementary polynucleotide tail and that will cleave the corresponding RNA by hydrolyzing its target site (namely "hammerhead ribozymes"). Briefly, the simplified cycle of a hammerhead ribozyme comprises (1) sequence specific binding to the target RNA via complementary antisense sequences; (2) site-specific hydrolysis of the cleavable motif of the target strand; and (3) release of cleavage products, which gives rise to another catalytic cycle. Indeed, the use of long-chain antisense polynucleotide (at least 30 bases long) or ribozymes with long antisense arms are advantageous. A preferred delivery system for antisense ribozyme is achieved by covalently linking these antisense ribozymes to lipophilic groups or to use liposomes as a convenient vector. Preferred antisense ribozymes according to the present invention are prepared as described by Sczakiel *et al.*(1995), the specific preparation procedures being referred to in said article being herein incorporated by reference.

Other methods of inhibiting SPANK expression known in the art may also be used, including homologous recombination and zinc fingers.

XI. Recombinant non-Human Animals of the Invention

The terms "transgenic animals" or "host animals" or "recombinant non-human animals" are used herein to designate animals that have their genome genetically and artificially manipulated so as to include one of the nucleic acids according to the invention. Preferred animals are non-human mammals and include those belonging to a genus selected from *Mus* (*e.g.* mice), *Rattus* (*e.g.* rats) and *Oryctogalus* (*e.g.* rabbits) which have their genome artificially and genetically altered by the insertion of a nucleic acid according to the invention. In one embodiment, the invention encompasses non-human host mammals and animals comprising a recombinant vector of the invention or a SPANK gene disrupted by homologous recombination with a knock out vector.

The transgenic animals of the invention all include within a plurality of their cells a cloned recombinant or synthetic DNA sequence, more specifically one of the purified or isolated nucleic acids comprising a SPANK coding sequence, a SPANK regulatory polynucleotide or a DNA sequence encoding an antisense polynucleotide such as described in the present specification.

Preferred transgenic animals according to the invention contain in their somatic cells and/or in their germ line cells a polynucleotide selected from the following group of polynucleotides:

a) a purified or isolated nucleic acid encoding a SPANK polypeptide, or a polypeptide fragment or variant thereof;

b) a purified or isolated nucleic comprising at least 8, at least 15, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, at least 300, or at least 500 consecutive nucleotides of the nucleotide sequence of SEQ ID No 2 or a variant thereof or a sequence complementary thereto;

c) a purified or isolated nucleic acid comprising at least 8 consecutive nucleotides, at least 15, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, at least 300, or at least 500 of the nucleotide sequence of SEQ ID No 3 or a variant thereof or a sequence complementary thereto;

d) a purified or isolated nucleic acid comprising at least 8 consecutive nucleotides, at least 15, at least 25, at least 50, at least 75, at least 100, at least 150, at least 200, at least 250, at least 300, or at least 500 of the nucleotide sequence of SEQ ID No 5 or a variant thereof or a sequence complementary thereto;

e) a purified or isolated nucleic acid encoding the polypeptide sequence SEQ ID No 1 or the sequences complementary thereto or a biologically active fragment or a variant thereof;

f) a purified or isolated nucleic acid encoding the polypeptide sequence SEQ ID No 4, or the sequence complementary thereto or a biologically active fragment or a variant thereof;

g) a purified or isolated nucleic acid encdng the polypeptide sequence SEQ ID No 6, or the sequence complementary thereto or a biologically active fragment or a variant thereof; and

h) a DNA construct as described previously in the present specification.

The transgenic animals of the invention thus contain specific sequences of exogenous genetic material such as the nucleotide sequences described above in detail.

In a first preferred embodiment, these transgenic animals may be good experimental models in order to study the diverse pathologies related to insulin resistance, in particular concerning the transgenic animals within the genome of which has been inserted one or several copies of a polynucleotide encoding a native SPANK protein, or alternatively a mutant SPANK protein.

In a second preferred embodiment, these transgenic animals may express a desired polypeptide of interest leading to good yields in the synthesis of this protein of interest, and eventually a tissue specific expression of this protein of interest.

The design of the transgenic animals of the invention may be made according to the conventional techniques well known from the one skilled in the art. For more details regarding the production of transgenic animals, and specifically transgenic mice, it may be referred to US Patents Nos 4,873,191, issued Oct. 10, 1989, 5,464,764 issued Nov 7, 1995 and 5,789,215, issued Aug 4, 1998, these documents being herein incorporated by reference to disclose methods producing transgenic mice.

Transgenic animals of the present invention are produced by the application of procedures that result in an animal with a genome that has incorporated exogenous genetic material. The procedure involves obtaining the genetic material, or a portion thereof, which encodes either a SPANK coding sequence, a SPANK regulatory polynucleotide or a DNA sequence encoding a SPANK antisense polynucleotide such as described in the present specification.

A recombinant polynucleotide of the invention is inserted into an embryonic or ES stem cell line. The insertion is preferably made using electroporation, such as described by Thomas *et al.*(1987). The cells subjected to electroporation are screened (*e.g.* by selection via selectable markers, by PCR or by Southern blot analysis) to find positive cells which have integrated the exogenous recombinant polynucleotide into their genome, preferably via an homologous recombination event. An illustrative positive-negative selection procedure that may be used according to the invention is described by Mansour *et al.*(1988).

Then, the positive cells are isolated, cloned and injected into 3.5 days old blastocysts from mice, such as described by Bradley (1987). The blastocysts are then inserted into a female host animal and allowed to grow to term.

Alternatively, the positive ES cells are brought into contact with embryos at the 2.5 days old 8-16 cell stage (morulae) such as described by Wood *et al.*(1993) or by Nagy *et al.*(1993), the ES cells being internalized to colonize extensively the blastocyst including the cells which will give rise to the germ line.

The offspring of the female host are tested to determine which animals are transgenic *e.g.* include the inserted exogenous DNA sequence and which are wild-type. Thus, the present invention also concerns a transgenic animal containing a nucleic acid, a recombinant expression vector or a recombinant host cell according to the invention.

A further object of the invention consists of recombinant host cells obtained from a transgenic animal described herein. In one embodiment the invention encompasses cells derived from non-human host mammals and animals comprising a recombinant vector of the invention or a SPANK gene disrupted by homologous recombination with a knock out vector.

Recombinant cell lines may be established *in vitro* from cells obtained from any tissue of a transgenic animal according to the invention, for example by transfection of primary cell cultures with vectors expressing *onc*-genes such as SV40 large T antigen, as described by Chou (1989) and Shay *et al.*(1991).

Other characteristics and advantages of the invention are described in the Examples. These are meant to be exemplary only, and not to limit the invention in any way. Throughout this application, various publications, patents and published patent applications are cited. The disclosures of these publications, patents and published patent specifications referenced in this application are hereby incorporated by reference into the present disclosure.

EXAMPLES

Example 1: Isolation of SPANK

SPANK was isolated using IRAP as bait in a two-hybrid yeast system. In the well-established two-hybrid system, a known protein is used as a "bait" to search for its interacting partner, or "prey" protein(s). Specifically, the IRAP cytosolic domain (aa 1-109 was fused to the DNA-binding domain of the transcription factor GAL4 to generate the IRAP bait construct. IRAP is a vesicular glycoprotein that co-purifies and co-localizes with GLUT4 and undergoes translocation in response to insulin (Kandror et al (1994) J Biol Chem 269:30777-80; Ross et al (1996) J Biol Chem 271:3328-32). It is ubiquitously expressed and abundant in insulin-sensitive tissues, such as skeletal muscle and adipocytes. A library of human skeletal muscle cDNA was fused to the transactivating domain of GAL4 to generate the prey library (Clontech). Both bait and prey were introduced into a yeast reporter strain, where a selectable marker is turned on by specific bait-prey interaction (James et al (1996) genetics 144:1425-36).

Using this screening strategy, IRAP was found to specifically interact with proteins encoded by two distinct cDNA clones from the skeletal muscle cDNA library. These clones represent overlapping fragments of the SPANK gene, encoding aa 436-1166 and aa 153-598 of SPANK protein. These two cDNA clones were used as a probe to hybridize with a lambda gt11 cDNA library made from human skeletal muscles, which allowed the identification of additional 5' sequences. Sequences from these two cDNA clones were also used to BLAST the EST database (http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast?Jform=1), which allowed the identification of additional overlapping 3' expressed sequence tags (EST), which were arranged (contigged) to produce continuous sequence.

Two other fragments of SPANK were shown to interact with IRAP in the two hybrid system (aa 436-1088 and aa 436-957). All fragments contain the ANK domain.

Example 2: Confirmation of the Association of SPANK with IRAP *in vivo*

The interaction between IRAP and SPANK has been confirmed biochemically and morphologically. First, in BOSC cells overexpressing epitope-tagged IRAP and SPANK, IRAP was detected in the SPANK immunoprecipitant. Conversely, SPANK was detected in the IRAP immunoprecipitant.

In these experiments, BOSC cells were transfected with FLAG-tagged SPANK and myc-tagged IRAP constructs using Fugene reagents (Boehringer Mannheim) following the manufacturer's recommendation. Two days after transfection, the cells were washed in PBS and lysed in buffer A (200 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 7.5 μg/mL aprotinin, 0.5 mM PMSF, 1 mg/mL leupeptin, 200 μM sodium orthovanadate and 20 mM ß-glycerophosphate). Clarified lysates were incubated with either anti-FLAG affinity resin (Sigma) or with polyclonal anti-myc antibody (BAbCo) followed by protein G Sepharose beads. The immunoprecipitants were washed extensively in buffer A alternating with buffer A containing 500 mM NaCl, and then separated by SDS-PAGE. The protein gels were immunoblotted with anti-FLAG antibody for SPANK and anti-myc antibody for IRAP.

Example 3: Identification of the Region of IRAP that SPANK Binds *in vitro*

The binding of SPANK to IRAP was also confirmed *in vitro*, where IRAP1-109 as a GST fusion bound to full-length SPANK.

To narrow down the region in IRAP that binds to SPANK, GST fusion proteins containing various IRAP fragments were examined for their binding to SPANK. More specifically, full-length SPANK was [35S]-labeled using a coupled *in vitro* transcription/translation system (Promega). Bacterial vectors expressing GST fused C-terminally to various regions of human IRAP were generated by inserting the appropriate PCR products into the EcoRI site of pGEX-4T1 (Pharmacia). IRAP-GST fusion proteins were induced in *E. coli* and purified on glutathione Sepharose beads as described (Frangioni et al, 1993 Anal Biochem. 210:179-187), except that no detergents were used.

IRAP-GST fusion proteins (20 μg) were incubated with [35S]-labeled SPANK at 4 oC for 3 h in 100 μL buffer A (200 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 7.5 μg/mL aprotinin, 0.5 mM PMSF, 1 mg/mL leupeptin, 200 μM sodium orthovanadate and 20 mM ß-glycerophosphate). The precipitants were washed 4 times with 1 mL of buffer A prior to analysis in 6.5% SDS-PAGE. Binding between the GST-IRAP and SPANK was quantitated by radiodensitometry.

To demonstrate the binding specificity, SPANK was shown to not bind to GST protein, and GST-IRAP was shown to not bind to ankyrin G, which contains 24 ANK repeats. Through a reiterative process, GST fused to RQSPDG (IRAP 96-101) was found to be sufficient for binding to SPANK. Its binding efficiency is indistinguishable from GST fused to the entire cytosolic domain of IRAP. Therefore, genetic and biochemical data indicate that binding between IRAP and SPANK requires a specific region of IRAP (*i.e.*, no more than IRAP96-101) and the ANK domain of SPANK.

Example 4: Interactions with the Non-IRAP-Binding Domain of SPANK

To uncover proteins that interact with the non-IRAP binding region of SPANK (SPANK devoid of ANK repeats), a fragment of SPANK (aa 813-1,166) was used as a bait in a similar yeast two-hybrid system as described above (Example 1) to screen a skeletal muscle library (Clontech) for interacting proteins. This uncovered only one specific interaction, a cDNA that encodes myosin.

To confirm the myosin-SPANK interaction biochemically, purified GST-myosin fusion protein was shown to bind to [35S] labeled SPANK protein *in vitro*. The myosin can be heavy or light chain, muscle isoform or non-muscle isoform.

Example 5: SPANK Phosphorylation

To demonstrate the phosphorylation of SPANK upon insulin stimulation, 3T3-L1 adipocytes (8 - 16 days after induction of adipogenic differentiation) were serum starved for 6-15 hours and then stimulated with insulin (1 ug/mL) for 10 min. At 4 °C thereafter, cells were washed with cold PBS containing 3 mM niacinamide (Sigma) and lysed with buffer A [200 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 7.5 μg/mL aprotinin, 0.5 mM PMSF, 1 mg/mL leupeptin, 200 μM sodium orthovanadate and 20 mM ß-glycerophosphate] containing 3 mM niacinamide for 30 min. Lysates were clarified at 14,000 g for 10 min and incubated with Sepharose beads containing GST-IRAPaa78-109 (20 μg fusion protein /plate lysates) for 8-28 h. After 3 washes in buffer A, SPANK was resolved in 6.5% SDS-PAGE.

In some experiments, adipocytes were maintained in phosphate/pyruvate-free DME for 2 h and then labeled with [^{32}P]-Pi (300 μCi/mL) for 2 h to allow labeling of phosphoproteins. These experiments revealed that SPANK undergoes quantitative phosphorylation within 10 minutes of insulin stimulation. The phosphorylation is accompanied by a 10 kD increase in SPANK's apparent molecular weight (*i.e.*, a 10 kD gel mobility shift).

Example 6: SPANK Expression

The expression pattern of SPANK at the mRNA level was analyzed by Northern blots, which involve the use of a labeled human SPANK cDNA probe to hybridize with mRNA from a panel of human tissues (Clontech) that includes heart, brain, placenta, lung, liver, skeletal, muscle, kidney, and pancreas, as well as 3T3-L1 fibroblasts (mouse) and 3T3-L1 adipocytes (mouse). SPANK is expressed in all tissues examined, and the expression is higher in adipocytes than in fibroblasts. This reveals that SPANK expression is ubiquitous. It is abundantly expressed in insulin sensitive-tissues (skeletal muscle, heart, and adipocytes) and is induced during adipogenic differentiation of fibroblasts.

Example 7: SPANK Targeting

The intracellular targeting of SPANK has been performed in both COS cells and in adipocytes. For COS cell experiments, a DNA construct containing a CMV promoter expressing FLAG-tagged SPANK was transfected into COS-7 cells (ATCC) using Fugene reagents. After 36-48 hours, COS cells were rinsed in PBS, fixed in methanol (5 min) and treated with acetone (2 min) at -20 °C. At 20 °C thereafter, samples were blocked with goat globulin (200 μg/mL; Jackson) and incubated with anti-FLAG M2 antibody (~1 μg/mL; Sigma). Primary antibodies were detected with Cy3-conjugated goat anti-mouse (~1.2 μg/mL; Jackson Immuno), and samples were mounted in Vectashield medium containing DAPI (Vector Lab). The images were acquired on a Nikon E800 microscope using the OpenLab software.

For the adipocyte experiment, a retroviral vector expressing FLAG-tagged SPANK was stably introduced into 3T3-L1 fibroblasts. Following the induction of adipogenic differentiation, the cells were processed essentially as described for COS cells for immunofluorescence. These experiments show that SPANK is predominantly targeted to the perinuclear region in the cytosol. Indirect evidence suggests that it colocalizes with GLUT4 in / near the Golgi apparatus.

The targeting pattern of SPANK can also be determined in 3T3-L1 cells by confocal indirect immunofluorescence microscopy. In these cells SPANK is expected to be targeted to the Golgi region perinuclearly. The targeting of SPANK likely overlaps with a significant pool of GLUT4

Example 8: SPANK PARP Activity

To demonstrate the PARP activity of SPANK, SPANK was incubated with the PARP substrate, NAD (nicotinamide adenine dinucleotide)(up to 1 mM) at 37 °C for 10 min with in 60 μL of 150 mM NaCl, 50 mM Tris pH 8, 5 mM $MgCl_2$, 10 % glycerol, 1 mM DTT and 0.1 mM PMSF. This allows the ADP-ribose moiety from NAD to be polymerized onto PARP substrates, and the resulting polymer can be immunoblotted using a commercial antibody (BioMol). Alternatively, ^{32}P-labled NAD can be used to label the poly(ADP-ribose).

These experiments reveal that purified SPANK can poly(ADP-ribosyl)ate itself *in vitro*. It can also poly(ADP-ribosyl)ate purified GST-IRAP *in vitro*. The *in vitro* PARP activity of SPANK can be inhibited by inhibitors such as niacinamide, aminobenzamide, and benzamide. Other inhibitors of PARP activity are known in the art and are expected to inhibit SPANK PARP activity as well.

Example 9: Effect of the Inhibition of PARP Activity

The PARP activity of SPANK appears to play a role in insulin-induced GLUT4 translocation, because brief treatment of adipocytes with niacinamide results in significant impairment of insulin-induced GLUT4 translocation. In these experiments, a GLUT4 construct containing multiple myc epitopes in the extracellular domains was stably expressed in 3T3-L1 adipocytes. The cells were serum starved and pre-treated with or without 30 mM niacinamide for 30 min. The cells were then stimulated with up to 160 nM of insulin for 5 min.

Intact cells with stained with anti-myc antibody to quantitate the GLUT4 that was translocated to cell surface. This revealed that niacinamide inhibits most of the insulin-induced translocation of GLUT4.

SPANK is unique in that it has been shown to physically interact with a component of GLUT4 vesicles. Using known methods, SPANK's binding to GLUT4, either directly or through an IRAP bridge can be assessed.

Reference for poly(ADP-ribose) glycohydrolase (which reverses PARP product) and its inhibitors (page 4, line 26) Aoki K *et al.*, 1993. Biochimica et Biophysica Acta, 1158, 251-256

Example 10: SPANK Antibody

Polyclonal antibody against SPANK has been raised in rabbits using SPANK fragments aa 813-881 and aa 935-1020 fused to GST and the C-terminal 19 residues of SPANK, not fused to GST using standard techniques well-known in the art. All fragments of SPANK created antibodies that can immunoblot and immunoprecipitate SPANK.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims. Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the claims.

CLAIMS

What is claimed is:

1. Isolated DNA encoding a protein which binds IRAP and is an effector in the insulin signaling pathway in a eukaryotic cell.

2. Isolated DNA of claim 1 which is human DNA.

3. Isolated DNA comprising DNA selected from the group consisting of:

a) DNA having the nucleic acid sequence of SEQ ID NO.:2;

b) DNA having a nucleic acid sequence which is at least 80% identical to the nucleic acid sequence of SEQ ID NO.:2 and encodes a protein which binds IRAP;

c) DNA comprising a nucleic acid sequence which is at least 95% identical to the nucleic acid sequence of SEQ ID NO.:2 and encodes a protein which binds IRAP;

d) DNA which encodes the amino acid sequence of SEQ ID NO.:1;

e) DNA which hybridizes under high stringency conditions to the complement of SEQ ID NO.:2

f) DNA comprising the nucleic acid sequence of SEQ ID NO.:3;

g) DNA which encodes a protein comprising the amino acid sequence of SEQ ID NO.:4;

h) DNA which hybridizes under high stringency conditions to the complement of SEQ ID NO.: 3;

i) DNA comprising the nucleic acid sequence of SEQ ID NO: 5;

j) DNA which hybridizes under high stringency conditions to the complement of DNA comprising SEQ ID NO.: 5; and

k) DNA which encodes a protein comprising the amino acid sequence of SEQ ID NO.: 6.

4. Isolated DNA encoding a protein which comprises three domains, binds IRAP and is an effector in the insulin signaling pathway in a cell, wherein the three domains are:

a) an ANK domain;

b) a SAM domain; and

c) a PARP catalytic domain.

5. Isolated DNA of claim 4 which is human DNA.

6. Isolated protein selected from the group consisting of:

a) a protein of SEQ ID NO.:1;

b) a protein comprising the amino acid sequence of SEQ ID NO.:4; and

c) a protein comprising the amino acid sequence of SEQ ID NO.: 6.

7. Isolated human protein which comprises three domains, binds IRAP and participates in translocation of GLUT4 from the interior of a cell, wherein the three domains are:

a) an ANK domain;

b) a SAM domain; and

c) a PARP catalytic domain.

8. A method of enhancing GLUT4 translocation in a cell, comprising contacting the cell with a drug, under conditions whereby the drug enters the cell, wherein the drug acts upon SPANK protein and alters SPANK binding of GLUT4, whereby GLUT4 undergoes translocation in the cell.

9. A method of enhancing GLUT4 translocation in a cell, comprising contacting the cell with a drug, under conditions whereby the drug enters the cell, wherein the drug acts upon SPANK protein and prevents SPANK

from binding GLUT4, whereby SPANK binding of GLUT4 is interrupted and GLUT4 undergoes translocation in the cell.

10. A method of enhancing glucose uptake in cells in an individual, comprising administering to the individual a drug which alters SPANK binding of GLUT4 in cells of the individual, whereby GLUT4 undergoes translocation in the cell and is available for glucose uptake, whereby glucose uptake is enhanced in the individual.

11. A method of enhancing glucose uptake in cells in an individual, comprising administering to the individual a drug which prevents SPANK binding of GLUT4 in cells of the individual, whereby SPANK binding of GLUT4 is interrupted, GLUT4 undergoes translocation in the cell and is available for glucose uptake, whereby glucose uptake is enhanced in the individual.

12. A purified, isolated, or recombinant SPANK polypeptide that has an effect on cells, wherein said effect is selected from the group consisting of modulation of GLUT4 translocation, modulation of IRAP-binding, and modulation of myosin-binding.

13. A purified, isolated, or recombinant SPANK polypeptide that comprises at least 180 contiguous amino acids of the amino acid sequence set forth in the group selected from SEQ ID NO: 1, SEQ ID NO: 4, and SEQ ID NO: 6.

14. A purified, isolated, or recombinant SPANK polypeptide that is at least 82% identical to the SPANK polypeptide sequences selected from the group consisting SEQ ID NO: 1; SEQ ID NO: 4; and SEQ ID NO:6.

15. A purified, isolated, or recombinant polynucleotide encoding said SPANK polypeptide of any one of claims 12 through 14, or the complement of the polynucleotide encoding said SPANK of any one of claims 12 through 14.

16. A recombinant vector comprising said polynucleotide of claim 15.

17. A recombinant cell comprising said recombinant vector of claim 16.

18. A pharmaceutical composition comprising said SPANK polypeptide of any one of claims 12 through 14 and a pharmaceutically acceptable diluent.

19. A method of reducing body mass comprising providing to an individual in need of reducing body mass said pharmaceutical composition of claim 18.

20. A method of reducing glucose intolerance or insulin resistance comprising providing to an individual in need of reducing body mass said pharmaceutical composition of claim 18.

21. A method of preventing or treating an obesity-related disease or disorder comprising providing to an individual in need of such treatment said pharmaceutical composition of claim 18.

22. The method of claim 21, wherein said obesity-related disease or disorder is selected from the group consisting of obesity, cardiac insufficiency, coronary insufficiency, stroke, hypertension, atheromatous disease, atherosclerosis, high blood pressure, non-insulin-dependent diabetes, hyperlipidemia, hyperuricemia, and Syndrome X.

23. A method of preventing or treating a muscle-related disease or disorder comprising providing to an individual in need of such treatment said pharmaceutical composition of claim 18.

24. The method of claim 23, wherein said muscle-related disease or disorder is selected from the group consisting of muscular dystrophy, muscle atrophy, and muscle fatigue.

25. A method for identifying one or more compounds that modulates SPANK activity comprising:

a) contacting a cell with a candidate compound;

6) detecting a result selected from the group consisting of a modulation in GLUT4 translocation, a modulation in IRAP-binding, a modulation in myosin binding, a modulation in PARP activity, and a modulation in SPANK phosphorylation; and

c) wherein said result identifies said one or more compounds if said result differs from said result in the absence of said one or more compounds.

26. The method of claim 25, wherein said SPANK is human.

27. The method of claim 25, wherein said one or more compounds is selected from the group selected from SPANK polypeptide fragments and IRAP fragments.

28. The method of claim 25, wherein said one or more compounds is selected from the group consisting of peptides, peptide libraries, non-peptide libraries, peptoids, fatty acids, lipoproteins, medicaments, antibodies, mimetics and small molecules.

29. An isolated, purified, or recombinant polynucleotide comprising a contiguous span of at least 540 nucleotides of the nucleotide sequence selected from the group consisting of SEQ ID NO: 2, SEQ ID NO:3, and SEQ ID NO:5 and the complements thereof.

30. A recombinant vector comprising the polynucleotide of claim 19.

31. A host cell comprising the recombinant vector of claim 20.

32. A non-human host animal or mammal comprising the recombinant vector of claim 2.

33. A mammalian host cell comprising a recombinantly disrupted SPANK gene.

34. A non-human host mammal comprising a recombinantly disrupted SPANK gene.

35. An isolated or purified antibody composition that selectively binds to an epitope-containing fragment of a SPANK polypeptide of claim 13.

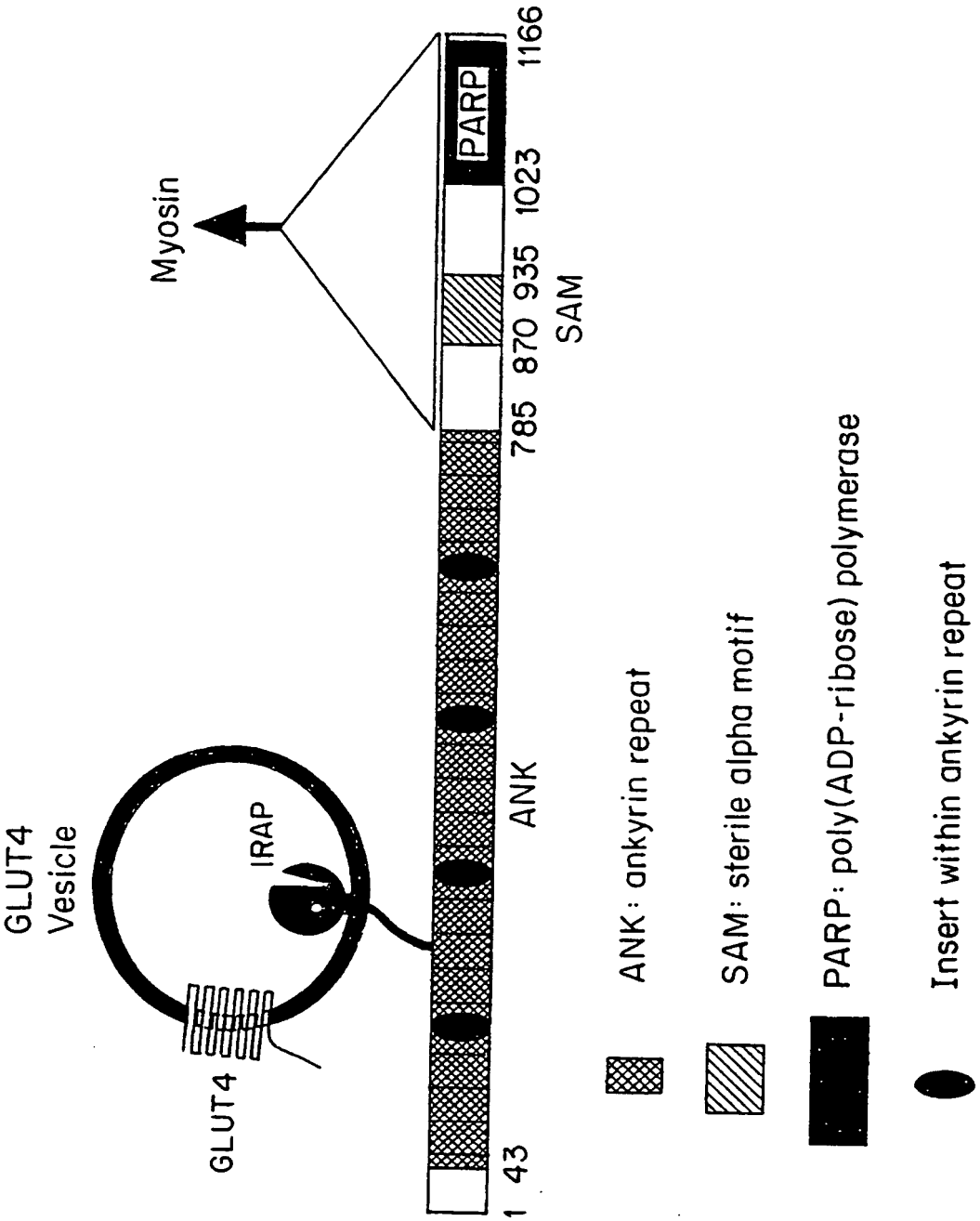

FIG. 1

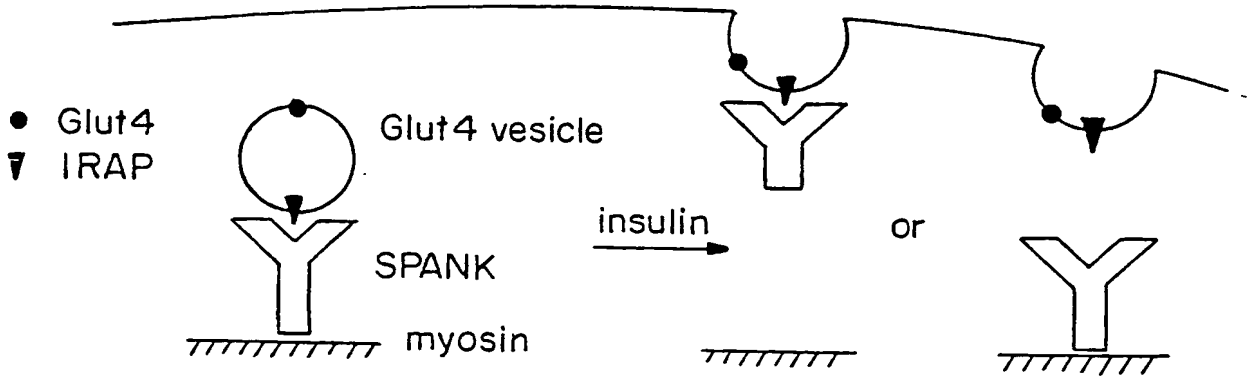

FIG. 2A

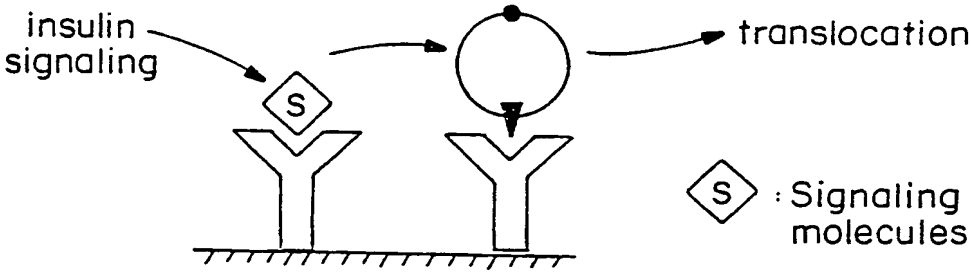

FIG. 2B

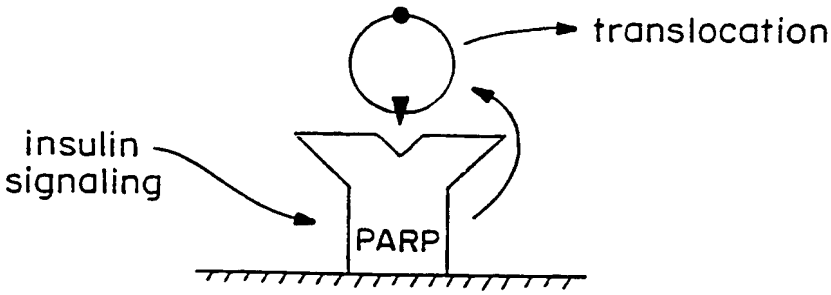

FIG. 2C

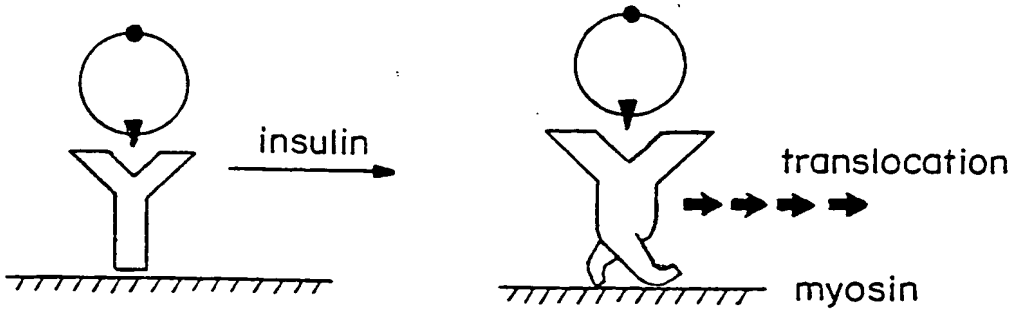

FIG. 2D

FIG 3A

Protein sequence of human SPANK as encoded by cDNA.

The ANK repeats are aligned, with shaded residues indicating identity to ANK repeat consensus. Sequences to the right indicate insertions that occur after the underlined residue in each line.

SAM domain is doubly underlined.

PARP domain is singly underlined.

FIG 3B

Human SPANK, cDNA sequence (Start and stop codons in bold):

CGTCGTTTCAGGACCCGGACGGCGGATTCGCGCTGCCTCCGCCGCCGCGGGGCAGC
CGGGGGGCAGGGAGCCCAGCGAGGGGCGCGCGTGGGCGCGGCCATGGGACTGCGC
CGGATCCGGTGACAGCAGGGAGCCAAGCGGCCCGGGCCCTGAGCGCGTCTTCTCCG
GGGGGCCTCGCCCTCCTGCTCGCGGGGCCGGGGCTCCTGCTCCGGTTGCTGGCGCTG
TTGCTGGCTGTGGCGGCGGCCAGGATC**ATG**TCGGGTCGCCGCTGCGCCGGCGGGGG
AGCGGCCTGCGCGAGCGCCGCGGCCGAGGCCGTGGAGCCGGCCGCCCGAGAGCTGT
TCGAGGCGTGCCGCAACGGGGACGTGGAACGAGTCAAGAGGCTGGTGACGCCTGAG
AAGGTGAACAGCCGCGACACGGCGGGCAGGAAATCCACCCCGCTGCACTTCGCCGC
AGGTTTTGGGCGGAAAGACGTAGTTGAATATTTGCTTCAGAATGGTGCAAATGTCCA
AGCACGTGATGATGGGGGCCTTATTCCTCTTCATAATGCATGCTCTTTTGGTCATGCT
GAAGTAGTCAATCTCCTTTTGCGACATGGTGCAGACCCCAATGCTCGAGATAATTGG
AATTATACTCCTCTCCATGAAGCTGCAATTAAAGGAAAGATTGATGTTTGCATTGTG
CTGTTACAGCATGGAGCTGAGCCAACCATCCGAAATACAGATGGAAGGACAGCATT
GGATTTAGCAGATCCATCTGCCAAAGCAGTGCTTACTGGTGAATATAAGAAAGATG
AACTCTTAGAAAGTGCCAGGAGTGGCAATGAAGAAAAAATGATGGCTCTACTCACA
CCATTAAATGTCAACTGCCACGCAAGTGATGGCAGAAAGTCAACTCCATTACATTTG
GCAGCAGGATATAACAGAGTAAAGATTGTACAGCTGTTACTGCAACATGGAGCTGA
TGTCCATGCTAAAGATAAAGGTGATCTGGTACCATTACACAATGCCTGTTCTTATGG
TCATTATGAAGTAACTGAACTTTTGGTCAAGCATGGTGCCTGTGTAAATGCAATGGA
CTTGTGGCAATTCACTCCTCTTCATGAGGCAGCTTCTAAGAACAGGGTTGAAGTATG
TTCTCTTCTCTTAAGTTATGGTGCAGACCCAACACTGCTCAATTGTCACAATAAAAGT
GCTATAGACTTGGCTCCCACACCACAGTTAAAAGAAAGATTAGCATATGAATTTAAA
GGCCACTCGTTGCTGCAAGCTGCACGAGAAGCTGATGTTACTCGAATCAAAAAACA
TCTCTCTCTGGAAATGGTGAATTTCAAGCATCCTCAAACACATGAAACAGCATTGCA
TTGTGCTGCTGCATCTCCATATCCCAAAAGAAAGCAAATATGTGAACTGTTGCTAAG
AAAAGGAGCAAACATCAATGAAAAGACTAAAGAATTCTTGACTCCTCTGCACGTGG
CATCTGAGAAAGCTCATAATGATGTTGTTGAAGTAGTGGTGAAACATGAAGCAAAG
GTTAATGCTCTGGATAATCTTGGTCAGACTTCTCTACACAGAGCTGCATATTGTGGTC
ATCTACAAACCTGCCGCCTACTCCTGAGCTATGGGTGTGATCCTAACATTATATCCCT
TCAGGGCTTTACTGCTTTACAGATGGGAAATGAAAATGTACAGCAACTCCTCCAAGA
GGGTATCTCATTAGGTAATTCAGAGGCAGACAGACAATTGCTGGAAGCTGCAAAGG

FIG 4A

CTGGAGATGTCGAAACTGTAAAAAAACTGTGTACTGTTCAGAGTGTCAACTGCAGA
GACATTGAAGGGCGTCAGTCTACACCACTTCATTTTGCAGCTGGGTATAACAGAGTG
TCCGTGGTGGAATATCTGCTACAGCATGGAGCTGATGTGCATGCTAAAGATAAAGG
AGGCCTTGTACCTTTGCACAATGCATGTTCTTATGGACATTATGAAGTTGCAGAACT
TCTTGTTAAACATGGAGCAGTAGTTAATGTAGCTGATTTATGGAAATTTACACCTTTA
CATGAAGCAGCAGCAAAAGGAAAATATGAAATTTGCAAACTTCTGCTCCAGCATGG
TGCAGACCCTACAAAAAAAAAACAGGGATGGAAATACTCCTTTGGATCTTGTTAAAG
ATGGAGATACAGATATTCAAGATCTGCTTAGGGGAGATGCAGCTTTGCTAGATGCTG
CCAAGAAGGGTTGTTTAGCCAGAGTGAAGAAGTTGTCTTCTCCTGATAATGTAAATT
GCCGCGATACCCAAGGCAGACATTCAACACCTTTACATTTAGCAGCTGGTTATAATA
ATTTAGAAGTTGCAGAGTATTTGTTACAACACGGAGCTGATGTGAATGCCCAAGACA
AAGGAGGACTTATTCCTTTACATAATGCAGCATCTTACGGGCATGTAGATGTAGCAG
CTCTACTAATAAAGTATAATGCATGTGTCAATGCCACGGACAAATGGGCTTTCACAC
CTTTGCACGAAGCAGCCCAAAAGGGACGAACACAGCTTTGTGCTTTGTTGCTAGCCC
ATGGAGCTGACCCGACTCTTAAAAATCAGGAAGGACAAACACCTTTAGATTTAGTTT
CAGCGGATGATGTCAGCGCTCTTCTGACAGCAGCCATGCCCCCATCTGCTCTGCCCT
CTTGTTACAAGCCTCAAGTGCTCAATGGTGTGAGAAGCCCAGGAGCCACTGCAGAT
GCTCTCTCTTCAGGTCCATCTAGCCCATCAAGCCTTTCTGCAGCCAGCAGTCTTGACA
ACTTATCTGGGAGTTTTTCAGAACTGTCTTCAGTAGTTAGTTCAAGTGGAACAGAGG
GTGCTTCCAGTTTGGAGAAAAAGGAGGTTCCAGGAGTAGATTTTAGCATAACTCAAT
TCGTAAGGAATCTTGGACTTGAGCACCTAATGGATATATTTGAGAGAGAACAGATCA
CTTTGGATGTATTAGTTGAGATGGGGCACAAGGAGCTGAAGGAGATTGGAATCAAT
GCTTATGGACATAGGCACAAACTAATTAAAGGAGTCGAGAGACTTATCTCCGGACA
ACAAGGTCTTAACCCATATTTAACTTTGAACACCTCTGGTAGTGGAACAATTCTTAT
AGATCTGTCTCCTGATGATAAAGAGTTTCAGTCTGTGGAGGAAGAGATGCAAAGTAC
AGTTCGAGAGCACAGAGATGGAGGTCATGCAGGTGGAATCTTCAACAGATACAATA
TTCTCAAGATTCAGAAGGTTTGTAACAAGAAACTATGGGAAAGATACACTCACCGG
AGAAAAGAAGTTTCTGAAGAAAACCACAACCATGCCAATGAACGAATGCTATTTCA
TGGGTCTCCTTTTGTGAATGCAATTATCCACAAAGGCTTTGATGAAAGGCATGCGTA
CATAGGTGGTATGTTTGGAGCTGGCATTTATTTTGCTGAAAACTCTTCCAAAAGCAA
TCAATATGTATATGGAATTGGAGGAGGTACTGGGTGTCCAGTTCACAAAGACAGATC
TTGTTACATTTGCCACAGGCAGCTGCTCTTTTGCCGGGTAACCTTGGGAAAGTCTTTC
CTGCAGTTCAGTGCAATGAAAATGGCACATTCTCCTCCAGGTCATCACTCAGTCACT
GGTAGGCCCAGTGTAAATGGCCTAGCATTAGCTGAATATGTTATTTACAGAGGAGAA
CAGGCTTATCCTGAGTATTTAATTACTTACCAGATTATGAGGCCTGAAGGTATGGTC
GATGGATAAATAGTTATTTTAAGAAACTAATTCCACTGAACCTAAAATCATCAAAGC
AGCAGTGGCCTCTACGTTTTACTCCTTTGCTGAAAAAAAAATCATCTTGCCCACAGGC
CTGTGGCAAAAGGATAAAAATGTGAACGAAGTTTAACATTCTGACTTGATAAAGCTT
TAATAATGTACAGTGTTTTCTAAATATTTCCTGTTTTTTCAGCACTTTAACAGATGCC
ATTCCAGGTTAAACTGGGTTGTCTGTACTAAATTATAAACAGAGTTAACTTGAACCT
TTTATATGTTATGCATTGATTCTAACAAACTGTAATGCCCTCAACAGAACTAATTTTA
CTAATACAATACTGTGTTCTTTAAAACACAGCATTTACACTGAATACAATTTCATTTG
TAAAACTGTAAATAAGAGCTTTTGTACTAGCCCAGTATTTATTTACATTGCTTTGTAA
TATAAATCTGTTTTAGAACTGCAGCGGTTTACAAAATTTTTTCATATGTATTGTTCAT
CTATACTTCATCTTACATCGTCATGATTGAGTGATCTTTACATTTGATTCCAGAGGCT

FIG 4B

ATGTTCAGTTGTTAGTTGGGAAAGATTGAGTTATCAGATTTAATTTGCCGATGGGAG
CCTTTATCTGTCATTAGAAATCTTTCTCATTTAAGAACTTATGAATATGCTGAAGATT
TAATTTGTGATACCTTTGTATGTATGAGACACATTCCAAAGAGCTCTAACTATGATA
ctgtgaaaattagagcaaagtgctcctgttttttagagaaactaaatcttgctgttgaacaattattgtgttcttttcatggaacataagtaggatgtt
acatttccagggtgggaagggtaatcctaaatcatttcccaatctattctaattaccttaaatctaaaggggaaaaaaaaaaatcacaaacaggac
tgggtagttttttatcctaagtatatttttttcctgttctttttacttggttttattgctgtatttatagccaatctatacatcatgggtaaacttaacccagaa
ctataaaatgtagttgtctcagtcccctccaggcctcctgaatgggcaagtgcagtgaaacaggtgcttcttgctcctgggttttctctccatgat
gttatgcccaattggaaatatgctgtcagtttgtgcaccatatggtgaccaggcctgtgctcagtttggcagctatagaaggaaatgctgtccca
taaaatgccattcctattttctaatataaaactcttttccaggaagcatgcttaagcatcttgttacagagacatacatccattatggcttggcaatct
ctttttatttgttgactctagctcccttcaaagtcgaggaaagatctttactcacttaatgaggacattccccatcactgtctgtaccagttcactttatt
ttacgttttattcagtctgtaaattaactggccctttgcagtaacttgtacataaagtgctagaaaatcatgttccttgtcctgagtaagagttaatca
gagtaaatgcatttctggagttgtttctgtgatgtaaattatgatcattatttaagaagtcaaatccctgatcttgaagtgctttttatacagctctctaa
taattacaaatatccgaaagtcatttcttggaacacaagtggagtatgccaaatttttatatgaatttttcagattatctaagcttccaggttttataatt
agaagataatgagagaattaatggggtttatatttacattatctctcaactatgtagcccatattactcaccctatgagtgaatctggaattgcttttc
atgtgaaatcattgtggtctatgagtttacaatactgcaaactgtgttatttttatctaatccattgcttaatgagtgtgtttttccatgaatgaatatacc
gtggttcatatgttagcatggcagcattttcagatagcttttgtttgttgggaagttggggttttgggggggaggggagtattagtacgttgcatg
aaatagcttactttataatgatggaattgcttttctttttgtcttgtgatttttttttttgaagtgaaatttaacttttgtgcaagtagtactattatacccat
cttcagtgtcttacttgtactgtatcacattccataccctcatttaattcttaataaaactgttcacttgtctttctgggtagcatggtaattactggaat
agtataaatgtgttgaatggtctttgagaaaatgaattaagattacaataaaccacaattgcagg

FIG 4C

Mouse SPANK cDNA, with gaps indicated by "--". The start codon and stop codons are in bold type.

GGCCCAGGACCCGACCGAGCTGGCAGGAGTGGTCTCGGCCGCTTCCGCCGTCGCGT
CGCTTCGGGGCTCGGCGAGCGGGTTCGCGCTGCCTCCACCGCGGTGGGGCAGCCGG
GGCTCACGGAGCCGAGCCGGGGGCGCGCGGCGACGCGGCCATGGGACCGTGCCGAC
TCTGGTGACAGCGGGGAGCGGAGCGGCCCGGGCCCTGAGCGCGTCTCCTGCGGGGG
GCCTCGCCCTCCTGCTCGCGGGGCCCGGGCTCCTGCTGCCTTTGCTGGCGCTGCTGCT
GGCGGCGGCGGCGGCGGCCCGGATCATGTCTGGCCGCCGCTGCGCCGGCGGGGGCG
CGGCCTGCGCGAGCGCCGGGGCCGAGGCCGTGGAGCCGTCCGCCCGCGAGCTGTTC
GAGGCTTGCCGCAACGGGGACGTGGAGCGAGTCAAGAGGCTGGTGACGCCCGAGA
AGGTGAACAGCCGCGACACGGCGGGCAGGAAGTCCACCCCGCTCCACTTCGCCGCA
GGTTTTGGACGGAAGGATGTAGTTGAATATCTTCTTCAGAACGGTGCAAATGTGCAA
GCGCGTGATGATGGGGGTCTTATTCCTCTTCATAACGCATGCTCTTTTGGTCATGCTG
AAGTAGTCAATCTTCTTTTGCAACACGGTGCAGACCCAAATGCTCGGGATAATTGGA
ATTATACTCCTCTCCATGAAGCTGCAATTAAAGGAAAAATTGATGTTTGCATTGTGC
TTTTACAGCATGGAGCTGAGCCAACCATCCGAAATACAGATGGAAGGACAGCATTG
GATTTAGCAGACCCATCTGCCAAAGCTGTTCTGACTGGTGACTATAAGAAAGATGAA
CTTCTGGAAAGTGCCAGGAGTGGCAATGAGGAAAAAATGATGGCCCTGCTTACACC

FIG 5A

ATTGAATGTCAACTGCCATGCAAGTGATGGCAGAAAGTCAACTCCATTGCATTTGGC
AGCAGGATACAACAGAGTAAAAATTGTACAACTGTTACTGCACCATGGGGCTGATG
TCCATGCTAAAGATAAAGGGGATCTGGTACCACTACACAATGCCTGCTCTTACGGTC
ATTATGAAGTAACTGAACTTCTGGTCAAGCACGGTGCCTGTGTGAATGCAATGGACC
TGTGGCAGTTCACTCCTCTTCATGAGGCAGCTTCTAAGAACAGGATTGAAGTATGTT
CTCTCCTCCTAAGTTACGGGGCAGATCCAACCCTGCTGAACTGTCACAATAAAAGTG
CTATAGACCTGGCCCCCACGGCACAGCTGAAGGAAAGATTATCGTATGAATTTAAA
GGCCATTCATTGCTGCAAGCTGCACGAGAAGCTGATGTTACAAGAATCAAAAAACA
TCTTTCTCTGGAAATGGTGAATTTCAAACATCCTCAGACACATGAAACAGCACTGCA
TTGTGCTGCTGCATCTCCATATCCCAAAAGAAAGCAGATATGTGAACTGCTGCTCAG
AAAGGGAGCAAACACCAACGAAAAGACTAAAGAATTCTTGACTCCTCTGCACGTGG
CTTCTGAGAACGCTCACAATGATGTTGTTGAAGTAGTGGTGAAACATGAAGCAAAG
GTTAATGCTTTGGATAGTCTTGGACAGACGTCATTACACAGAGCTGCACACTGTGGT
CACCTGCAGACCTGCCGCCTGCTCCTGAGCTATGGGTGTGATCCAAACATCATATCC
CTTCAGGGTTTCACCGCCTTGCAAATGGGAAATGAAAATGTGCAGCAGCTGCTCCAA
G---GAAACTGTAAAAAAAACTCTGTACTGTTCAGAGTGTCAACTGCAGAGACATTGAA
GGACGCCAGTCAACCCCACTCCACTTTGCAGCTGGGTACAACAGAGTGTCTGTGGTA
GAATATCTGCTGCAACATGGAGCTGATGTTCATGCTAAAGACAAAGGAGGTCTTGTA
CCTTTACACAATGCATGTTCTTATGGACACTATGAAGTTGCAGAACTTCTTGTCAAG
CATGGAGCAGTAGTCAATGTCGCTGACTTGTGGAAGTTCACACCTTTACATGAAGCT
GCAGCAAAAGGAAAATATGAAATATGCAAACTTCTACTCCAGCATGGTGCAGACCC
TACAAAGAAAAACAGAGATGGTAATACTCCTCTGGATCTTGTTAAAGATGGAG ---
GATGCAGCTTTGCTGGATGCTGCCAAGAAGGGTTGTTTAGCCAGAGTGAAGAAATTG
TCTTCTCCTGATAATGTAAATTGCCGTGATACCCAAGGTCGGCATTCTACACCTTTAC
ATTTGGCAGCTGGTTATAATAACTTGGAAGTTGCTGAGTATTTATTGCAACATGGAG
CTGATGTGAATGCCCAGGACAAAGGAGGACTCATTCCTTTACATAACGCAGCATCGT
ATGGACATGTAGATGTAGCAGCTTTGCTGATAAAGTACAATGCGTGTGTCAATGCCA
CGGACAAATGGGCTTTCACACCGTTACATGAAGCAGCCCAAAAGGGACGGACACAG
CTTTGTGCTTTATTGTTGGCCCATGGAGCTGATCCTACTCTTAAAAATCAAGAAGGA
CAAACACCTTTAGATTTAGTTTCAGCAGATGATGTCAGTGCACTCTTGACAGCAGCC
ATGCC
---GGAACAATTCTCATAGATCTGTCTCCTGATGATAAAGAATTTCAGTCTGTGGAAGA
AGAGATGCAGAGTACTGTGCGGGAGCACAGAGATGGTGGTCACGCAGGCGGCGTCT
TCAACAGATACAACATTCTCAAGATTCAGAAGGTTTGTAACAAGAAATTGTGGGAA
AGATATACACACCGGAGAAAAGAAGTTTCTGAAGAAAACCACAACCATGCAAACGA
AAGGATGTTATTTCATGGGTCTCCTTTTGTGAATGCGATTATCCATAAGGGCTTTGAT
GAAAGGCATGCATACATAGGTGGCATGTTTGGAGCTGGAATTTATTTTGCTGAGAAC
TCTTCCAAAAGCAATCAATATGTGTATGGAATTGGAGGTGGCACCGGATGTCCAATT
CACAAAGACAGATCGTGTTACATTTGTCACAGGCAGCTGCTGTTTTGTCGAGTAACC
TTGGGAAAGTCTTTCTTGCAGTTCAGCGCAATGAAAATGGCACATTCTCCTCCCGGC
CATCACTCGGTCACTGGCCGGCCCAGTGTAAATGGCCTAGCATTAGCTGAATATGTT
ATTTACAGAGGAGAACAGGCTTATCCTGAATATCTAATTACTTACCAGATTGTAAGG
CCTGAAGGTATGGTTGATGGCTGA

FIG 5A a

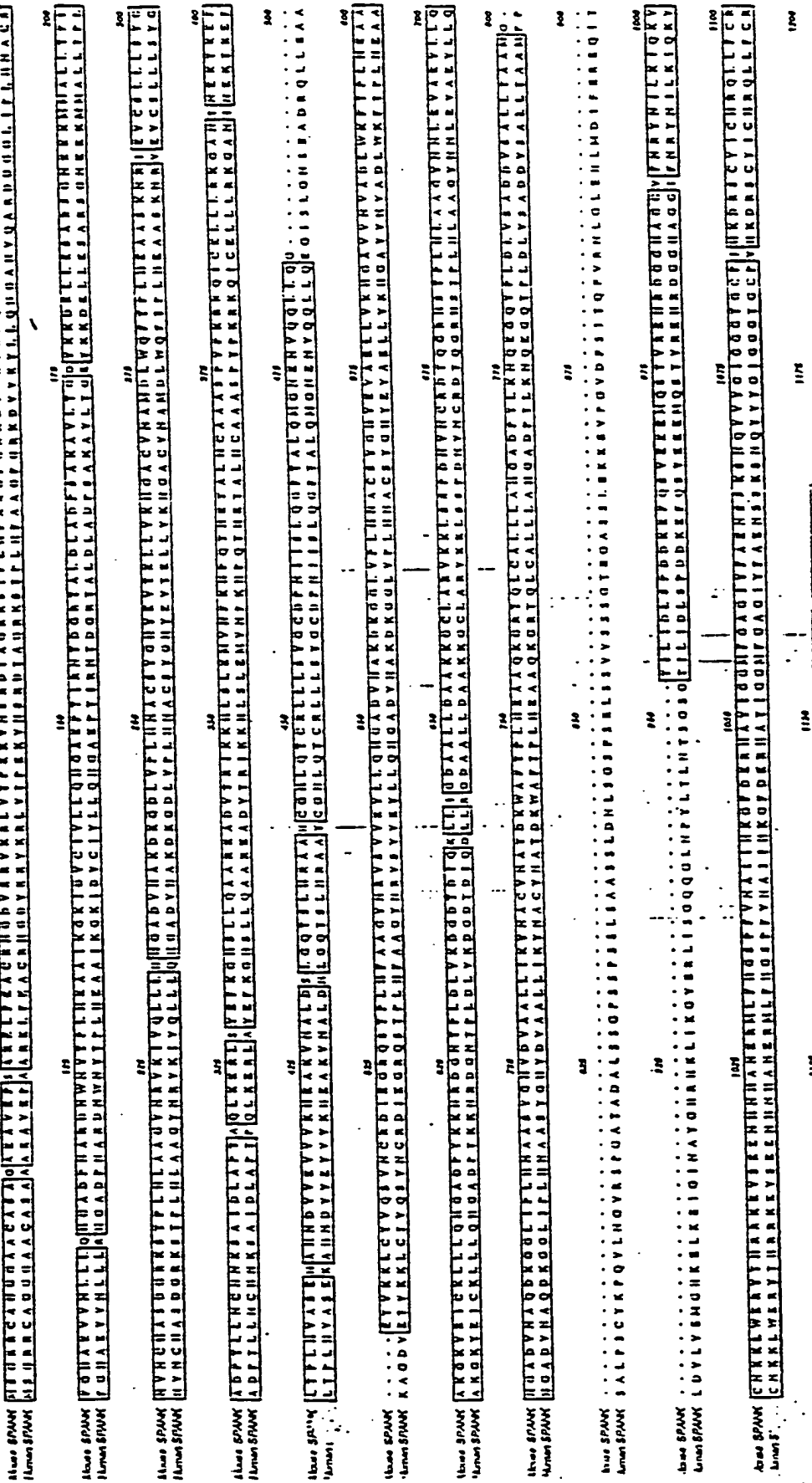

FIG 5B

Rat SPANK partial cDNA sequence, with "--" indicating gaps

GGAATAGATTTTAGTATAACTCAGTTCATAAGGAACCTTGGACTTGAGCATTTAATGGACATATTTG
AGCGAGAACAG--CAGTCTTTGGAAGAAGAGATGCAAAGTACTGTTCGAGAGCACAGGGATGGAGGT
CACGCAGGCGGCATCTTCAACAGATACAGTATTCTCAAGATTCAGAAGGTTTGTAACAAGAAATTGT
GGGAAAGATATACACACCGGAGAAAAGAAGTTTCTGAAGAAAACCACAACCATACAAATGAAAGAAT
GTTATTTCATGGT--AGACAAATGCTTTTCTTTAGAGTGACCCTTGGAAAGTCTTTTCTGCAATCCA
GCGCCATGAAAATGGCCCATGCGCCTCCAGGACATCACTCAGTCATCGGTAGACCAAGTGTCAATGG
GCTGGCGTATGCTGAATATGTCATCTACAGAGGGGAACAGGCATATGCAGAATATCTCATCACCTTC
CAGATCATGAAGCCGGAA

FIG 6

Rat SPANK partial peptide sequence, with "--" indicating ga
ps

GIDFSITQFIRNLGLEHLMDIFEREQ--QSLEEEMQSTVREHRDGGHAGGIFNRYSILKIQKVCNKK
LWERYTHRRKEVSEENHNHTNERMLFHG--
RQMLFFRVTLGKSFLQSSAMKMAHAPPGHHSVIGRPSVNGLAYAEYVIYRGEQAYAEYLITFQIMKP
E

FIG 7

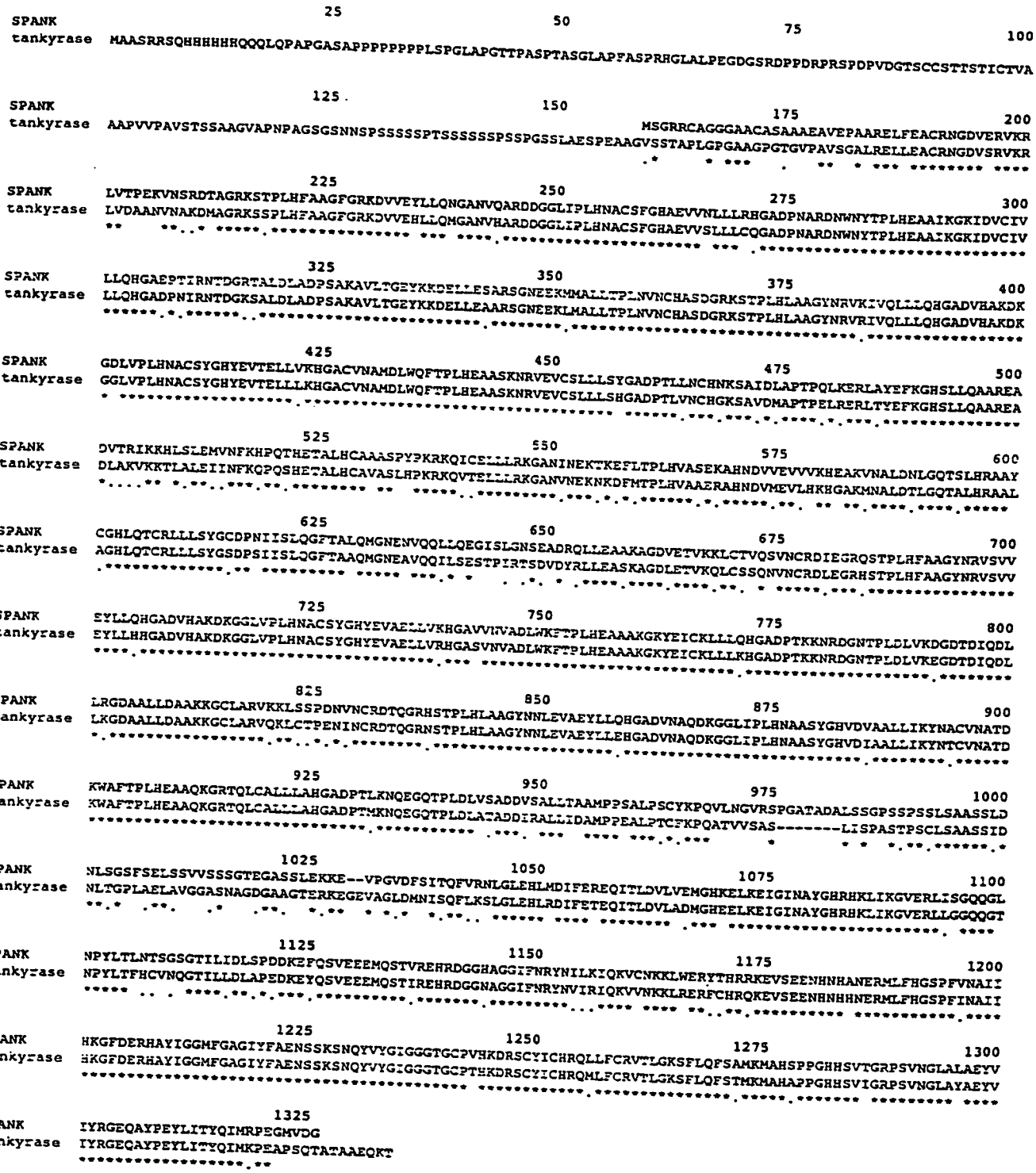
SPANK
tankyrase
MAASRRSQHHHHHHQQQLQPAPGASAPPPPPPPPLSPGLAPGTTPASPTASGLAPFASPRHGLALPEGDGSRDPPDRPRSPDPVDGTSCCSTTSTICTVA
AAPVVPAVSTSSAAGVAPNPAGSGSNNSPSSSSSPTSSSSSSPSSPGSSLAESPEAAGVSSTAPLGPGAAGPGTGVPAVSGALRELLEACRNGDVSRVKR
MSGRRCAGGGAACASAAAEAVEPAARELFEACRNGDVERVKR
LVTPEKVNSRDTAGRKSTPLHFAAGFGRKDVVEYLLQNGANVQARDDGGLIPLHNACSFGHAEVVNLLLRHGADPNARDNWNYTPLHEAAIKGKIDVCIV
LVDAANVNAKDMAGRKSSPLHFAAGFGRKDVVEHLLQMGANVHARDDGGLIPLHNACSFGHAEVVSLLLCQGADPNARDNWNYTPLHEAAIKGKIDVCIV
LLQHGAEPTIRNTDGRTALDLADPSAKAVLTGEYKKDELLESARSGNEEKMMALLTPLNVNCHASDGRKSTPLHLAAGYNRVKIVQLLLQHGADVHAKDK
LLQHGADPNIRNTDGKSALDLADPSAKAVLTGEYKKDELLEAARSGNEEKLMALLTPLNVNCHASDGRKSTPLHLAAGYNRVRIVQLLLQHGADVHAKDK
GDLVPLHNACSYGHYEVTELLVKHGACVNAMDLWQFTPLHEAASKNRVEVCSLLLSYGADPTLLNCHNKSAIDLAPTPQLKERLAYEFKGHSLLQAAREA
GGLVPLHNACSYGHYEVTELLLKHGACVNAMDLWQFTPLHEAASKNRVEVCSLLLSHGADPTLVNCHGKSAVDMAPTPELRERLTYEFKGHSLLQAAREA
DVTRIKKHLSLEMVNFKHPQTHETALHCAAASPYPKRKQICELLLRKGANINEKTKEFLTPLHVASEKAHNDVVEVVVKHEAKVNALDNLGQTSLHRAAY
DLAKVKKTLALEIINFKQPQSHETALHCAVASLHPKRKQVTELLLRKGANVNEKNKDFMTPLHVAAERAHNDVMEVLHKHGAKMNALDTLGQTALHRAAL
CGHLQTCRLLLSYGCDPNIISLQGFTALQMGNENVQQLLQEGISLGNSEADRQLLEAAKAGDVETVKKLCTVQSVNCRDIEGRQSTPLHFAAGYNRVSVV
AGHLQTCRLLLSYGSDPSIISLQGFTAAQMGNEAVQQILSESTPIRTSDVDYRLLEASKAGDLETVKQLCSSQNVNCRDLEGRHSTPLHFAAGYNRVSVV
EYLLQHGADVHAKDKGGLVPLHNACSYGHYEVAELLVKHGAVVNVADLWKFTPLHEAAAKGKYEICKLLLQHGADPTKKNRDGNTPLDLVKDGDTDIQDL
EYLLHHGADVHAKDKGGLVPLHNACSYGHYEVAELLVRHGASVNVADLWKFTPLHEAAAKGKYEICKLLLKHGADPTKKNRDGNTPLDLVKEGDTDIQDL
LRGDAALLDAAKKGCLARVKKLSSPDNVNCRDTQGRHSTPLHLAAGYNNLEVAEYLLQHGADVNAQDKGGLIPLHNAASYGHVDVAALLIKYNACVNATD
LKGDAALLDAAKKGCLARVQKLCTPENINCRDTQGRNSTPLHLAAGYNNLEVAEYLLEHGADVNAQDKGGLIPLHNAASYGHVDIAALLIKYNTCVNATD
KWAFTPLHEAAQKGRTQLCALLLAHGADPTLKNQEGQTPLDLVSADDVSALLTAAMPPSALPSCYKPQVLNGVRSPGATADALSSGPSSPSSLSAASSLD
KWAFTPLHEAAQKGRTQLCALLLAHGADPTMKNQEGQTPLDLATADDIRALLIDAMPPEALPTCFKPQATVVSAS-------LISPASTPSCLSAASSID
NLSGSFSELSSVVSSSGTEGASSLEKKE--VPGVDFSITQFVRNLGLEHLMDIFEREQITLDVLVEMGHKELKEIGINAYGHRHKLIKGVERLISGQQGL
NLTGPLAELAVGGASNAGDGAAGTERKEGEVAGLDMNISQFLKSLGLEHLRDIFETEQITLDVLADMGHEELKEIGINAYGHRHKLIKGVERLLGGQQGT
NPYLTLNTSGSGTILIDLSPDDKEFQSVEEEMQSTVREHRDGGHAGGIFNRYNILKIQKVCNKKLWERYTHRRKEVSEENHNHANERMLFHGSPFVNAII
NPYLTFHCVNQGTILLDLAPEDKEYQSVEEEMQSTIREHRDGGNAGGIFNRYNVIRIQKVVNKKLRERFCHRQKEVSEENHNHHNERMLFHGSPFINAII
HKGFDERHAYIGGMFGAGIYFAENSSKSNQYVYGIGGGTGCPVHKDRSCYICHRQLLFCRVTLGKSFLQFSAMKMAHSPPGHHSVTGRPSVNGLALAEYV
HKGFDERHAYIGGMFGAGIYFAENSSKSNQYVYGIGGGTGCPTHKDRSCYICHRQMLFCRVTLGKSFLQFSTMKMAHAPPGHHSVIGRPSVNGLAYAEYV
IYRGEQAYPEYLITYQIMRPEGMVDG
IYRGEQAYPEYLITYQIMKPEAPSQTATAAEQKT

FIG 8

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 00/15926

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/54 C12N9/10 A61K38/45 C12Q1/48 G01N33/50
A01K67/027 C07K16/40 //A61P3/00,21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K C07K C12Q G01N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 15647 A (GARVAN INST MED RES ;SUTHERLAND ROBERT LYNDSAY (AU); DALY ROGER JO) 1 April 1999 (1999-04-01) abstract page 9, line 25 -page 10, line 2 claims	1-7, 12-18, 29-31,35
X	LEE WAN ET AL: "A synthetic peptide corresponding to the GLUT4 C-terminal cytoplasmic domain causes insulin-like glucose transport stimulation and GLUT4 recruitment in rat adipocytes." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 34, 1997, pages 21427-21431, XP002152802 ISSN: 0021-9258 the whole document	8-11,25, 28

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 2000	30/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Andres, S

3

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 00/15926

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WATERS STEVEN B ET AL: "The amino terminus of insulin-responsive aminopeptidase causes Glut4 translocation in 3T3-L1 adipocytes." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 37, 1997, pages 23323-23327, XP002152803 ISSN: 0021-9258 the whole document	8,9,25, 27,28
X	SMITH S ET AL: "Tankyrase, a poly(ADP-ribose) polymerase at human telomeres" SCIENCE, vol. 282, no. 5393, 20 November 1998 (1998-11-20), pages 1484-1487, XP002118903 ISSN: 0036-8075 the whole document	1-7
X	BALDINI G ET AL: "Insulin and nonhydrolyzable GTP analogs induce translocation of GLUT 4 to the plasma membrane in alpha-toxin-permeabilized rat adipose cells." JOURNAL OF BIOLOGICAL CHEMISTRY, (1991 MAR 5) 266 (7) 4037-40. , XP002152804 the whole document	8,9
A	ROSS STUART A ET AL: "Increased intracellular sequestration of the insulin-regulated aminopeptidase upon differentiation of 3T3-L1 cells." BIOCHEMICAL JOURNAL, vol. 330, no. 2, 1 March 1998 (1998-03-01), pages 1003-1008, XP002152805 ISSN: 0264-6021	
P,X	WO 99 64606 A (SMITH SUSAN ;LANGE TITIA DE (US); UNIV ROCKEFELLER (US)) 16 December 1999 (1999-12-16) page 6, line 13 -page 10 page 67 -page 94	1-7

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intr ional Application No
PCT/US 00/15926

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9915647 A	01-04-1999	AU 9245898 A	12-04-1999
		EP 1017802 A	12-07-2000
WO 9964606 A	16-12-1999	AU 4430199 A	30-12-1999

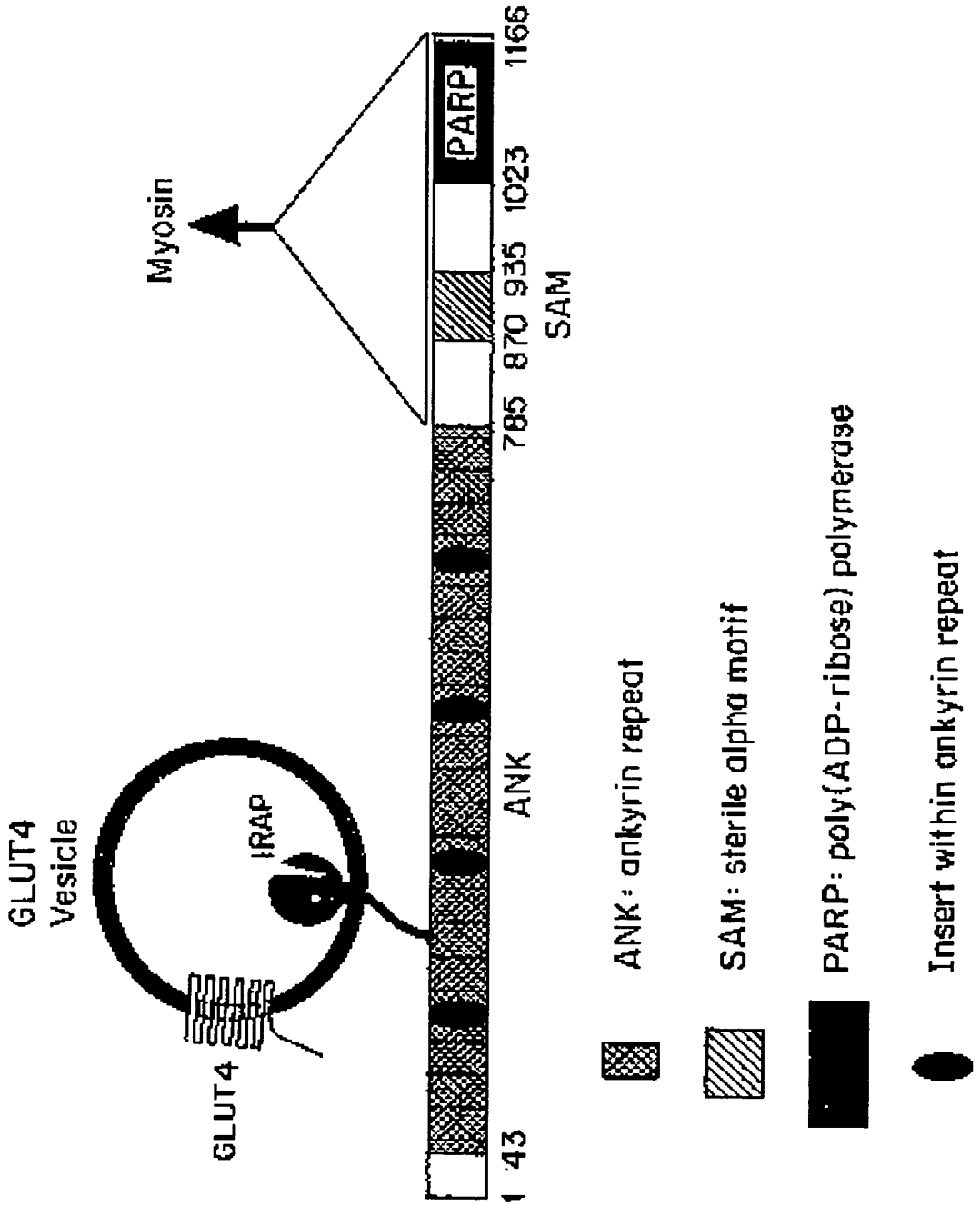

FIG. 1

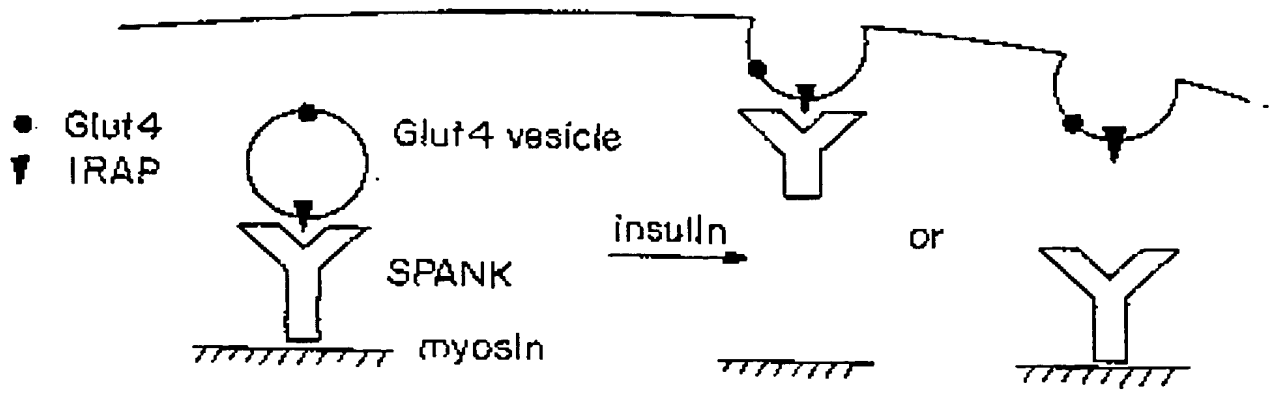

FIG. 2A

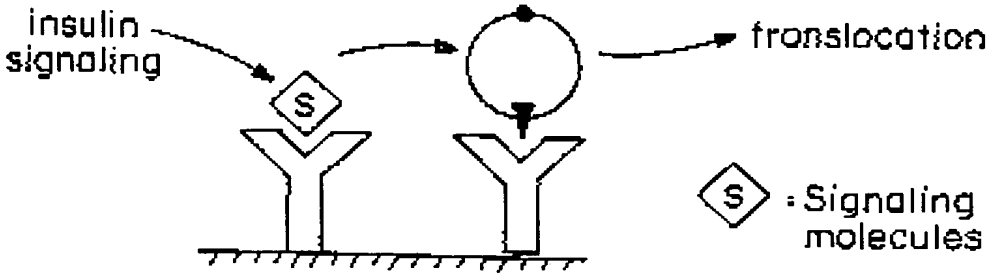

FIG. 2B

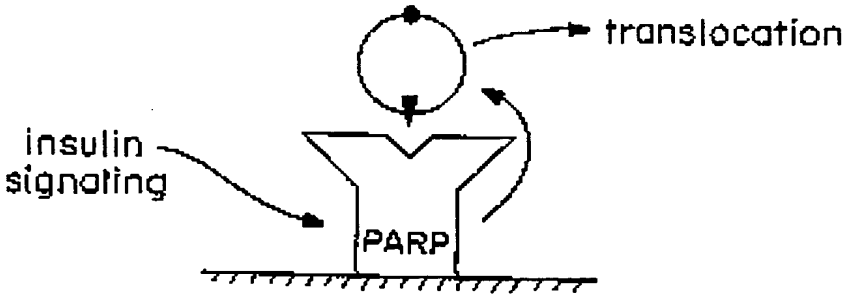

FIG. 2C

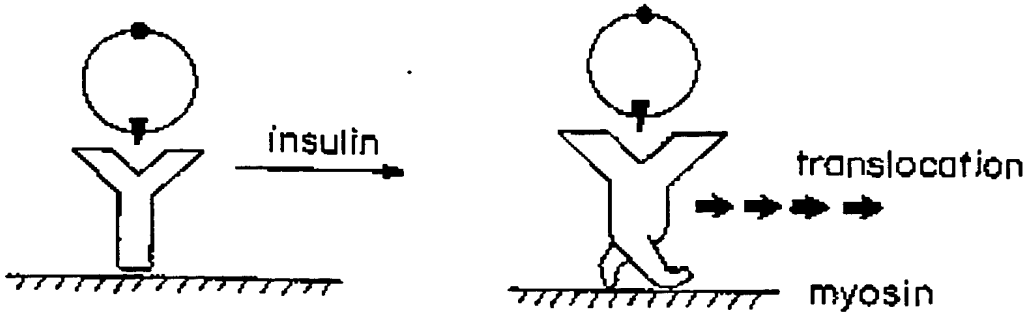

FIG. 2D

1 MSGRRCAGGGAACASAAAEAV
22 EPAARELFEACRNGDVERVKR[redacted]VTPEK[redacted]SRETAG
57 RKS[redacted]AGF[redacted]RKD[redacted]EYL[redacted]QNGAN[redacted]RED
90 G[redacted]LI[redacted]N[redacted]CSF[redacted]HAE[redacted]NL[redacted]RII[redacted]PL[redacted]REN
123 WNY[redacted]E[redacted]AIK[redacted]KID[redacted]CIVL[redacted]QHGAEPTIRNT
156 D[redacted]R[redacted]A[redacted]DPSAKAVLKMMA[redacted]LTPLN[redacted]CHASDG TGEYKKDELLESARSGNEE
210 RKS[redacted]AGYNRVK[redacted]QL[redacted]QH[redacted]HPKDK
243 GDLV[redacted]N[redacted]CSY[redacted]HYE[redacted]TE[redacted]VKHGAC[redacted]MDL
276 WQF[redacted]E[redacted]ASKNRVE[redacted]CS[redacted]SY[redacted]PTLLNC
309 HNKSAID[redacted]PTPQLKERLIKKHLSLEM[redacted]FKHPQ AYEFKGHSLLQAAREADVTR
363 THE[redacted]C[redacted]AASKRKQICE[redacted]RK[redacted]NI[redacted]EKTK PYP
399 EFL[redacted]V[redacted]SEKAHND[redacted]EVVVKHI[redacted]K[redacted]LN
432 L[redacted]QI[redacted]RE[redacted]AYC[redacted]HLQTCR[redacted]SY[redacted]IISL
465 Q[redacted]A[redacted]QMGNENVQQLLTVKK[redacted]CTVQS[redacted]CR[redacted]IEG QEGISLGNSEADRQLLEAAKAGDVE
525 RQS[redacted]AAGYNRVS[redacted]EY[redacted]QH[redacted]HPKDK
558 G[redacted]LV[redacted]N[redacted]CSY[redacted]HYE[redacted]AE[redacted]VKHG[redacted]VSSVA[redacted]L
591 WKF[redacted]E[redacted]AAK[redacted]KYEICK[redacted]QH[redacted]PTKKNR
624 D[redacted]N[redacted]D[redacted]VKDGDTDIQRVKK[redacted]SSPDN[redacted]CR[redacted]TQG DLLRGDAALLDAAKKGCLA
678 RHS[redacted]AGYNNLE[redacted]AEY[redacted]QH[redacted]QLK
711 G[redacted]LI[redacted]N[redacted]ASY[redacted]HVD[redacted]AA[redacted]IKYN[redacted]T[redacted]K
744 WAF[redacted]EAAQK[redacted]RTQLCA[redacted]AH[redacted]PTLKNQ
777 E[redacted]Q[redacted]D[redacted]VSADDVSALLTAAMPPSALPSCYKPQVLNGVRSPGATADALSSGPSSPSSLSAASSL
842 DNLSGSFSELSSVVSSSGTEGASSLEKK

870 EVPGVDFSITQFVRNLGLEHLMDIFEREQITLDVLVEMGHKELKEIGINAYGHRHKLIKGVERLIS

936 GQQGLNPYLTLNTSGSGTILIDLSPDDKEFQSVEEEMQSTVREII
980 RDGGHAGGIFNRYNILKIQKVCNKKLWERYTHRRKEVSEENHN

1023 HANERMLFHGSPFVNAIIHKGFDERHAYIGGMFGAGIYFAENSSKSNQYVYGIGGGTGCPVHKDRSCYICHR
1095 QLLFCRVTLGKSFLQFSAMKMAHSPPGIHSVTGRPSVNGLALAEYVIYRGEQAYPEYLITYQIMRPEGMVDG

FIG 3A

Protein sequence of human SPANK as encoded by cDNA.

The ANK repeats are aligned, with shaded residues indicating identity to ANK repeat consensus. Sequences to the right indicate insertions that occur after the underlined residue in each line.

SAM domain is doubly underlined.

PARP domain is singly underlined.

FIG 3B

Human SPANK, cDNA sequence (Start and stop codons in bold):

CGTCGTTTCAGGACCCGGACGGCGGATTCGCGCTGCCTCCGCCGCCGCGGGGCAGC
CGGGGGGCAGGGAGCCCAGCGAGGGGCGCGCGTGGGCGCGGCCATGGGACTGCGC
CGGATCCGGTGACAGCAGGGAGCCAAGCGGCCCGGGCCCTGAGCGCGTCTTCTCCG
GGGGCCCTCGCCCTCCTGCTCGCGGGGCCGGGGCTCCTGCTCCGGTTGCTGGCGCTG
TTGCTGGCTGTGGCGGCGGCCAGGATCATGTCGGGTCGCCGCTGCGCCGGCGGGGGG
AGCGGCCTGCGCGAGCGCCGCGGCCGAGGCCGTGGAGCCGGCCGCCCGAGAGCTGT
TCGAGGCGTGCCGCAACGGGGACGTGGAACGAGTCAAGAGGCTGGTGACGCCTGAG
AAGGTGAACAGCCGCGACACGGCGGGCAGGAAATCCACCCCGCTGCACTTCGCCGC
AGGTTTTGGGCGGAAAGACGTAGTTGAATATTTGCTTCAGAATGGTGCAAATGTCCA
AGCACGTGATGATGGGGGCCTTATTCCTCTTCATAATGCATGCTCTTTTGGTCATGCT
GAAGTAGTCAATCTCCTTTTGCGACATGGTGCAGACCCCAATGCTCGAGATAATTGG
AATTATACTCCTCTCCATGAAGCTGCAATTAAAGGAAAGATTGATGTTTGCATTGTG
CTGTTACAGCATGGAGCTGAGCCAACCATCCGAAATACAGATGGAAGGACAGCATT
GGATTTAGCAGATCCATCTGCCAAAGCAGTGCTTACTGGTGAATATAAGAAAGATG
AACTCTTAGAAAGTGCCAGGAGTGGCAATGAAGAAAAAATGATGGCTCTACTCACA
CCATTAAATGTCAACTGCCACGCAAGTGATGGCAGAAAGTCAACTCCATTACATTTG
GCAGCAGGATATAACAGAGTAAAGATTGTACAGCTGTTACTGCAACATGGAGCTGA
TGTCCATGCTAAAGATAAAGGTGATCTGGTACCATTACACAATGCCTGTTCTTATGG
TCATTATGAAGTAACTGAACTTTTGGTCAAGCATGGTGCCTGTGTAAATGCAATGGA
CTTGTGGCAATTCACTCCTCTTCATGAGGCAGCTTCTAAGAACAGGGTTGAAGTATG
TTCTCTTCTCTTAAGTTATGGTGCAGACCCAACACTGCTCAATTGTCACAATAAAAGT
GCTATAGACTTGGCTCCCACACCACAGTTAAAAGAAAGATTAGCATATGAATTTAAA
GGCCACTCGTTGCTGCAAGCTGCACGAGAAGCTGATGTTACTCGAATCAAAAAACA
TCTCTCTCTGGAAATGGTGAATTTCAAGCATCCTCAAACACATGAAACAGCATTGCA
TTGTGCTGCTGCATCTCCATATCCCAAAAGAAAGCAAATATGTGAACTGTTGCTAAG
AAAAGGAGCAAACATCAATGAAAAGACTAAAGAATTCTTGACTCCTCTGCACGTGG
CATCTGAGAAAGCTCATAATGATGTTGTTGAAGTAGTGGTGAAACATGAAGCAAAG
GTTAATGCTCTGGATAATCTTGGTCAGACTTCTCTACACAGAGCTGCATAITGTGGTC
ATCTACAAACCTGCCGCCTACTCCTGAGCTATGGGTGTGATCCTAACATTATATCCCT
TCAGGGCTTTACTGCTTTACAGATGGGAAATGAAAATGTACAGCAACTCCTCCAAGA
GGGTATCTCATTAGGTAATTCAGAGGCAGACAGACAATTGCTGGAAGCTGCAAAGG

FIG 4A

CTGGAGATGTCGAAACTGTAAAAAAACTGTGTACTGTTCAGAGTGTCAACTGCAGA
GACATTGAAGGGCGTCAGTCTACACCACTTCATTTTGCAGCTGGGTATAACAGAGTG
TCCGTGGTGGAATATCTGCTACAGCATGGAGCTGATGTGCATGCTAAAGATAAAGG
AGGCCTTGTACCTTTGCACAATGCATGTTCTTATGGACATTATGAAGTTGCAGAACT
TCTTGTTAAACATGGAGCAGTAGTTAATGTAGCTGATTTATGGAAATTTACACCTTTA
CATGAAGCAGCAGCAAAAGGAAAATATGAAATTTGCAAACTTCTGCTCCAGCATGG
TGCAGACCCTACAAAAAAAAAACAGGGATGGAAATACTCCTTTGGATCTTGTTAAAG
ATGGAGATACAGATATTCAAGATCTGCTTAGGGGAGATGCAGCTTTGCTAGATGCTG
CCAAGAAGGGTTGTTTAGCCAGAGTGAAGAAGTTGTCTTCTCCTGATAATGTAAATT
GCCGCGATACCCAAGGCAGACATTCAACACCTTTACATTTAGCAGCTGGTTATAATA
ATTTAGAAGTTGCAGAGTATTTGTTACAACACGGAGCTGATGTGAATGCCCAAGACA
AAGGAGGACTTATTCCTTTACATAATGCAGCATCTTACGGGCATGTAGATGTAGCAG
CTCTACTAATAAAGTATAATGCATGTGTCAATGCCACGGACAAATGGGCTTTCACAC
CTTTGCACGAAGCAGCCCAAAAGGGACGAACACAGCTTTGTGCTTTGTTGCTAGCCC
ATGGAGCTGACCCGACTCTTAAAAATCAGGAAGGACAAACACCTTTAGATTTAGTTT
CAGCGGATGATGTCAGCGCTCTTCTGACAGCAGCCATGCCCCCATCTGCTCTGCCCT
CTTGTTACAAGCCTCAAGTGCTCAATGGTGTGAGAAGCCCAGGAGCCACTGCAGAT
GCTCTCTCTTCAGGTCCATCTAGCCCATCAAGCCTTTCTGCAGCCAGCAGTCTTGACA
ACTTATCTGGGAGTTTTTCAGAACTGTCTTCAGTAGTTAGTTCAAGTGGAACAGAGG
GTGCTTCCAGTTTGGAGAAAAAGGAGGTTCCAGGAGTAGATTTTAGCATAACTCAAT
TCGTAAGGAATCTTGGACTTGAGCACCTAATGGATATATTTGAGAGAGAACAGATCA
CTTTGGATGTATTAGTTGAGATGGGGCACAAGGAGCTGAAGGAGATTGGAATCAAT
GCTTATGGACATAGGCACAAACTAATTAAAGGAGTCGAGAGACTTATCTCCGGACA
ACAAGGTCTTAACCCATATTTAACTTTGAACACCTCTGGTAGTGGAACAATTCTTAT
AGATCTGTCTCCTGATGATAAAGAGTTTCAGTCTGTGGAGGAAGAGATGCAAAGTAC
AGTTCGAGAGCACAGAGATGGAGGTCATGCAGGTGGAATCTTCAACAGATACAATA
TTCTCAAGATTCAGAAGGTTTGTAACAAGAAACTATGGGAAAGATACACTCACCGG
AGAAAAGAAGTTTCTGAAGAAAACCACAACCATGCCAATGAACGAATGCTATTTCA
TGGGTCTCCTTTTGTGAATGCAATTATCCACAAAGGCTTTGATGAAAGGCATGCGTA
CATAGGTGGTATGTTTGGAGCTGGCATTTATTTTGCTGAAAACTCTTCCAAAAGCAA
TCAATATGTATATGGAATTGGAGGAGGTACTGGGTGTCCAGTTCACAAAGACAGATC
TTGTTACATTTGCCACAGGCAGCTGCTCTTTTGCCGGGTAACCTTGGGAAAGTCTTTC
CTGCAGTTCAGTGCAATGAAAATGGCACATTCTCCTCCAGGTCATCACTCAGTCACT
GGTAGGCCCAGTGTAAATGGCCTAGCATTAGCTGAATATGTTATTTACAGAGGAGAA
CAGGCTTATCCTGAGTATTTAATTACTTACCAGATTATGAGGCCTGAAGGTATGGTC
GATGGATAAATAGTTATTTTAAGAAACTAATTCCACTGAACCTAAAATCATCAAAGC
AGCAGTGGCCTCTACGTTTTACTCCTTTGCTGAAAAAAAAATCATCTTGCCCACAGGC
CTGTGGCAAAAGGATAAAAATGTGAACGAAGTTTAACATTCTGACTTGATAAAGCTT
TAATAATGTACAGTGTTTTCTAAATATTTCCTGTTTTTTCAGCACTTTAACAGATGCC
ATTCCAGGTTAAACTGGGTTGTCTGTACTAAATTATAAACAGAGTTAACTTGAACCT
TTTATATGTTATGCATTGATTCTAACAAACTGTAATGCCCTCAACAGAACTAATTTTA
CTAATACAATACTGTGTTCTTTAAAACACAGCATTTACACTGAATACAATTTCATTTG
TAAAACTGTAAATAAGAGCTTTTGTACTAGCCCAGTATTTATTTACATTGCTTTGTAA
TATAAATCTGTTTTAGAACTGCAGCGGTTTACAAAATTTTTTCATATGTATTGTTCAT
CTATACTTCATCTTACATCGTCATGATTGAGTGATCTTTACATTTGATTCCAGAGGCT

FIG 4B

ATGTTCAGTTGTTACTTGGGAAAGATTGAGTTATCAGATTTAATTTGCCGATGGGAG
CCTTTATCTGTCATTAGAAATCTTTCTCATTTAAGAACTTATGAATATGCTGAAGATT
TAATTTGTGATACCTTTGTATGTATGAGACACATTCCAAAGAGCTCTAACTATGATA
GGTCCTGATTACTAAAGAAGCTTCTTTACTGGCCtcaatttctagctttcatgttggaaaatttctgcagtcctt
ctgtgaaaattagagcaaagtgctcctgttttttagagaaactaaatcttgctgttgaacaattattgtgttcttttcatggaacataagtaggatgtt
acatttccagggtgggaagggtaatcctaaatcatttcccaatctattctaattaccttaaatctaaaggggaaaaaaaaaaatcacaaacaggac
tgggtagttttttatcctaagtatatttttcctgttcttttacttggttttattgctgtatttatagccaatctatacatcatgggtaaacttaacccagaa
ctataaaatgtagttgtctcagtcccctccaggcctcctgaatgggcaagtgcagtgaaacaggtgcttcttgctcctgggttttctctccatgat
gttatgcccaattggaaatatgctgtcagttgtgcaccatatggtgaccaggcctgtgctcagtttggcagctatagaaggaaatgctgtccca
taaaatgccatttctatttctaatataaaactcttttccaggaagcatgcttaagcatcttgttacagagacatacatccattatggcttggcaatct
cttttattgttgactctagctcccttcaaagtcgaggaaagatctttactcacttaatgaggacatttcccatcactgtctgtacccagttcactttatt
ttacgttttattcagtctgtaaattaactggccctttgcagtaacttgtacataaagtgctagaaaatcatgttccttgtcctgagtaagagttaatca
gagtaaatgcatttctggagttgtttctgtgatgtaaattatgatcattatttaagaagtcaaatcctgatcttgaagtgctttttatacagctctctaa
taatttacaaatatccgaaagtcatttcttggaacacaagtggagtatgccaaattttatatgaatttttcagattatctaagcttccaggttttataatt
agaagataatgagagaattaatggggtttatatttacattatctctcaactatgtagccccatattactcacccctatgagtgaatctggaattgctttc
atgtgaaatcattgtggtctatgagtttacaatactgcaaactgtgttatttatctaatccattgcttaatgagtgtgttttttccatgaatgaatatacc
gtggttcatatgttagcatggcagcatttcagatagctttttgtttgttgggaagttggggttttgggggggaggggggagtattagtacgttgcatg
aaatagcttacttttataatgatggaattgctttttcttttgtcttgtgattttttttttgaagtgaaatttaacttttgtgtgcaagtagtactattataccccat
cttcagtgtcttacttgtactgtatcacattccatacccctcatttaattcttaataaaactgttcacttgtcttttctgggtagcatggtaattactggaat
agtataaatgtgttgaatggtcttgagaaaatgaattaagattacaataaaccacaattgcagg

FIG 4C

Mouse SPANK cDNA, with gaps indicated by "--". The start codon and stop codons are in bold type.

GGCCCAGGACCCGACCGAGCTGGCAGGAGTGGTCTCGGCCGCTTCCGCCGTCGCGT
CGCTTCGGGGCTCGGCGAGCGGGTTCGCGCTGCCTCCACCGCGGTGGGGCAGCCGG
GGCTCACGGAGCCGAGCCGGGGGCGCGCGGCGACGCGGCCATGGGACCGTGCCGAC
TCTGGTGACAGCGGGGAGCGGAGCGGCCCGGGCCCTGAGCCGCGTCTCCTGCGGGGG
GCCTCGCCCTCCTGCTCGCGGGGCCCGGGCTCCTGCTGCCTTTGCTGGCGCTGCTGCT
GGCGGCGGCGGCGGCGGCCCGGATCATGTCTGGCCGCCGCTGCGCCGGCGGGGGCG
CGGCCTGCGCCGAGCGCCGGGGCCGAGGCCGTGGAGCCGTCCGCCCGCGAGCTGTTC
GAGGCTTGCCCGCAACGGGGACGTGGAGCGAGTCAAGAGGCTGGTGACGCCCGAGA
AGGTGAACAGCCGCGACACGGCGGGCAGGAAGTCCACCCCGCTCCACTTCGCCGCA
GGTTTTGGACGGAAGGATGTAGTTGAATATCTTCTTCAGAACGGTGCAAATGTGCAA
GCGCGTGATGATGGGGGTCTTATTCCTCTTCATAACGCATGCTCTTTTGGTCATGCTG
AAGTAGTCAATCTTCTTTTGCAACACGGTGCAGACCCAAATGCTCGGGATAATTGGA
ATTATACTCCTCTCCATGAAGCTGCAATTAAAGGAAAAATTGATGTTTGCATTGTGC
TTTTACAGCATGGAGCTGAGCCAACCATCCGAAATACAGATGGAAGGACAGCATTG
GATTTAGCAGACCCATCTGCCAAAGCTGTTCTGACTGGTGACTATAAGAAAGATGAA
CTTCTGGAAAGTGCCAGGAGTGGCAATGAGGAAAAAATGATGGCCCTGCTTACACC

FIG 5A

ATTGAATGTCAACTGCCATGCAAGTGATGGCAGAAAGTCAACTCCATTGCATTTGGC
AGCAGGATACAACAGAGTAAAAATTGTACAACTGTTACTGCACCATGGGGCTGATG
TCCATGCTAAAGATAAAGGGGATCTGGTACCACTACACAATGCCTGCTCTTACGGTC
ATTATGAAGTAACTGAACTTCTGGTCAAGCACGGTGCCTGTGTGAATGCAATGGACC
TGTGGCAGTTCACTCCTCTTCATGAGGCAGCTTCTAAGAACAGGATTGAAGTATGTT
CTCTCCTCCTAAGTTACGGGGCAGATCCAACCCTGCTGAACTGTCACAATAAAAGTG
CTATAGACCTGGCCCCCACGGCACAGCTGAAGGAAAGATTATCGTATGAATTTAAA
GGCCATTCATTGCTGCAAGCTGCACGAGAAGCTGATGTTACAAGAATCAAAAAACA
TCTTTCTCTGGAAATGGTGAATTTCAAACATCCTCAGACACATGAAACAGCACTGCA
TTGTGCTGCTGCATCTCCATATCCCAAAAGAAAGCAGATATGTGAACTGCTGCTCAG
AAAGGGAGCAAACACCAACGAAAAGACTAAAGAATTCTTGACTCCTCTGCACGTGG
CTTCTGAGAACGCTCACAATGATGTTGTTGAAGTAGTGGTGAAACATGAAGCAAAG
GTTAATGCTTTGGATAGTCTTGGACAGACGTCATTACACAGAGCTGCACACTGTGGT
CACCTGCAGACCTGCCGCCTGCTCCTGAGCTATGGGTGTGATCCAAACATCATATCC
CTTCAGGGTTTCACCGCCTTGCAAATGGGAAATGAAAATGTGCAGCAGCTGCTCCAA
G--GAAACTGTAAAAAAAACTCTGTACTGTTCAGAGTGTCAACTGCAGAGACATTGAA
GGACGCCAGTCAACCCCACTCCACTTTGCAGCTGGGTACAACAGAGTGTCTGTGGTA
GAATATCTGCTGCAACATGGAGCTGATGTTCATGCTAAAGACAAAGGAGGTCTTGTA
CCTTTACACAATGCATGTTCTTATGGACACTATGAAGTTGCAGAACTTCTTGTCAAG
CATGGAGCAGTAGTCAATGTCGCTGACTTGTGGAAGTTCACACCTTTACATGAAGCT
GCAGCAAAAGGAAAATATGAAATATGCAAACTTCTACTCCAGCATGGTGCAGACCC
TACAAAGAAAAACAGAGATGGTAATACTCCTCTGGATCTTGTTAAAGATGGAG ---
GATGCAGCTTTGCTGGATGCTGCCAAGAAGGGTTGTTTAGCCAGAGTGAAGAAATTG
TCTTCTCCTGATAATGTAAATTGCCGTGATACCCAAGGTCGGCATTCTACACCTTTAC
ATTTGGCAGCTGGTTATAATAACTTGGAAGTTGCTGAGTATTTATTGCAACATGGAG
CTGATGTGAATGCCCAGGACAAAGGAGGACTCATTCCTTTACATAACGCAGCATCGT
ATGGACATGTAGATGTAGCAGCTTTGCTGATAAAGTACAATGCGTGTGTCAATGCCA
CGGACAAATGGGCTTTCACACCGTTACATGAAGCAGCCCAAAAGGGACGGACACAG
CTTTGTGCTTTATTGTTGGCCCATGGAGCTGATCCTACTCTTAAAAATCAAGAAGGA
CAAACACCTTTAGATTTAGTTTCAGCAGATGATGTCAGTGCACTCTTGACAGCAGCC
ATGCC
—GGAACAATTCTCATAGATCTGTCTCCTGATGATAAAGAATTTCAGTCTGTGGAAGA
AGAGATGCAGAGTACTGTGCGGGAGCACAGAGATGGTGGTCACGCAGGCGGCGTCT
TCAACAGATACAACATTCTCAAGATTCAGAAGGTTTGTAACAAGAAATTGTGGGAA
AGATATACACACCGGAGAAAAGAAGTTTCTGAAGAAAACCACAACCATGCAAACGA
AAGGATGTTATTTCATGGGTCTCCTTTTGTGAATGCGATTATCCATAAGGGCTTTGAT
GAAAGGCATGCATACATAGGTGGCATGTTTGGAGCTGGAATTTATTTTGCTGAGAAC
TCTTCCAAAAGCAATCAATATGTGTATGGAATTGGAGGTGGCACCGGATGTCCAATT
CACAAAGACAGATCGTGTTACATTTGTCACAGGCAGCTGCTGTTTTGTCGAGTAACC
TTGGGAAAGTCTTTCTTGCAGTTCAGCGCAATGAAAATGGCACATTCTCCTCCCGGC
CATCACTCGGTCACTGGCCGGCCCAGTGTAAATGGCCTAGCATTAGCTGAATATGTT
ATTTACAGAGGAGAACAGGCTTATCCTGAATATCTAATTACTTACCAGATTGTAAGG
CCTGAAGGTATGGTTGATGGCTGA

FIG 5A a

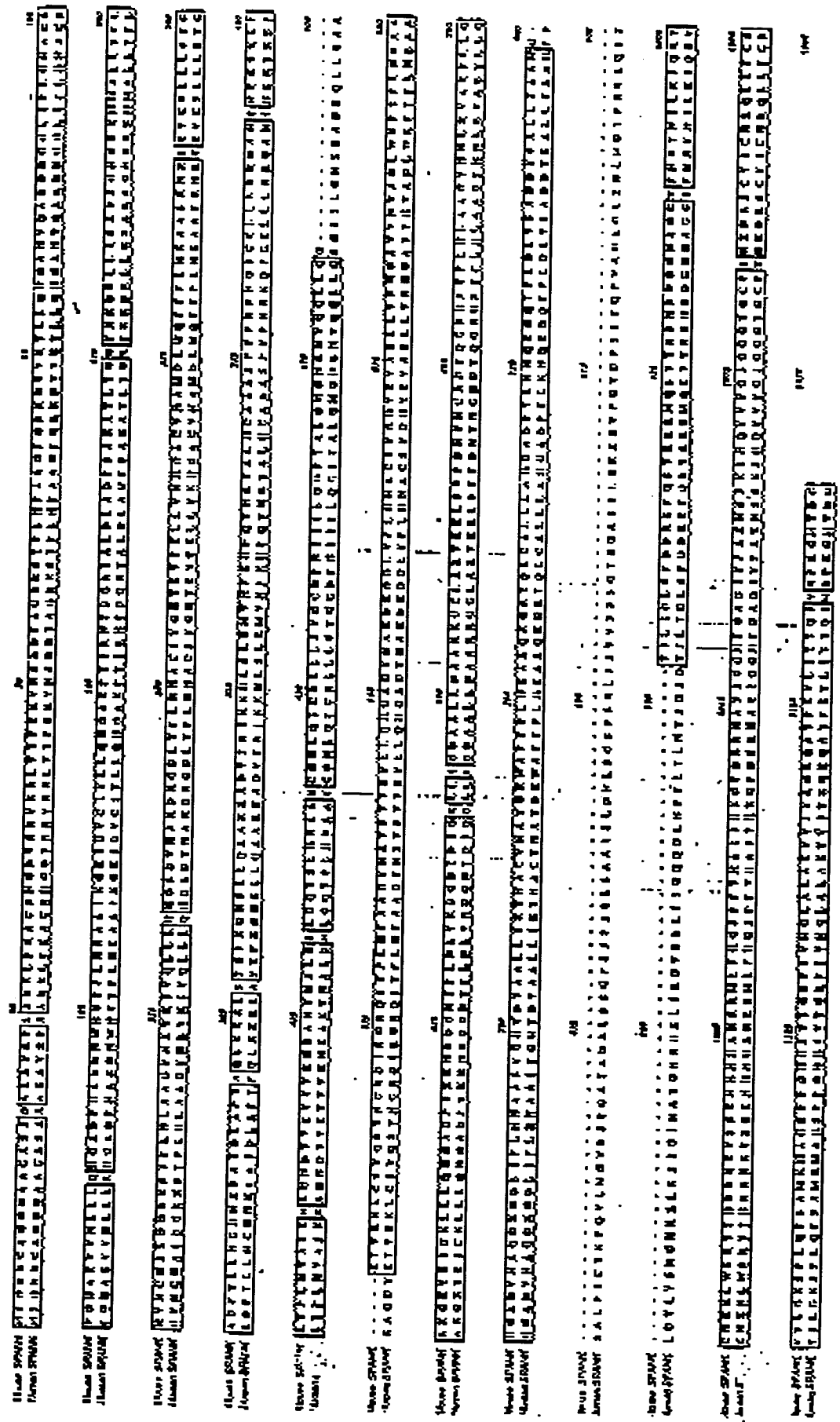

FIG 5B

Rat SPANK partial cDNA sequence, with "--" indicating gaps

GGAATAGATTTTAGTATAACTCAGTTCATAAGGAACCTTGGACTTGAGCATTTAATGGACATATTTG
AGCGAGAACAG--CAGTCTTTGGAAGAAGAGATGCAAAGTACTGTTCGAGAGCACAGGGATGGAGGT
CACGCAGGCGGCATCTTCAACAGATACAGTATTCTCAAGATTCAGAAGGTTTGTAACAAGAAATTGT
GGGAAAGATATACACACCGGAGAAAAGAAGTTTCTGAAGAAAACCACACAACCATACAAATGAAAGAAT
GTTATTTCATGGT--AGACAAATGCTTTTCTTTAGAGTGACCCTTGGAAAGTCTTTTCTGCAATCCA
GCGCCATGAAAATGGCCCATGCGCCTCCAGGACATCACTCAGTCATCGGTAGACCAAGTGTCAATGG
GCTGGCGTATGCTGAATATGTCATCTACAGAGGGGAACAGGCATATGCAGAATATCTCATCACCTTC
CAGATCATGAAGCCGGAA

FIG 6

Rat SPANK partial peptide eequence, with "--" indicating gaps

GIDFSITQFIRNLGLEHLMDIFEREQ--QSLEEEMQSTVREHRDGGHAGGIFNRYSILKIQKVCNKK
LWERYTHRRKEVSEENHNHTNERMLFHG--
RQMLPFRVTLGKSPLQSSAMKMAHAPPGHHSVIGRPSVNGLAYAEYVIYRGEQAYAEYLITFQIMKP
E

FIG 7

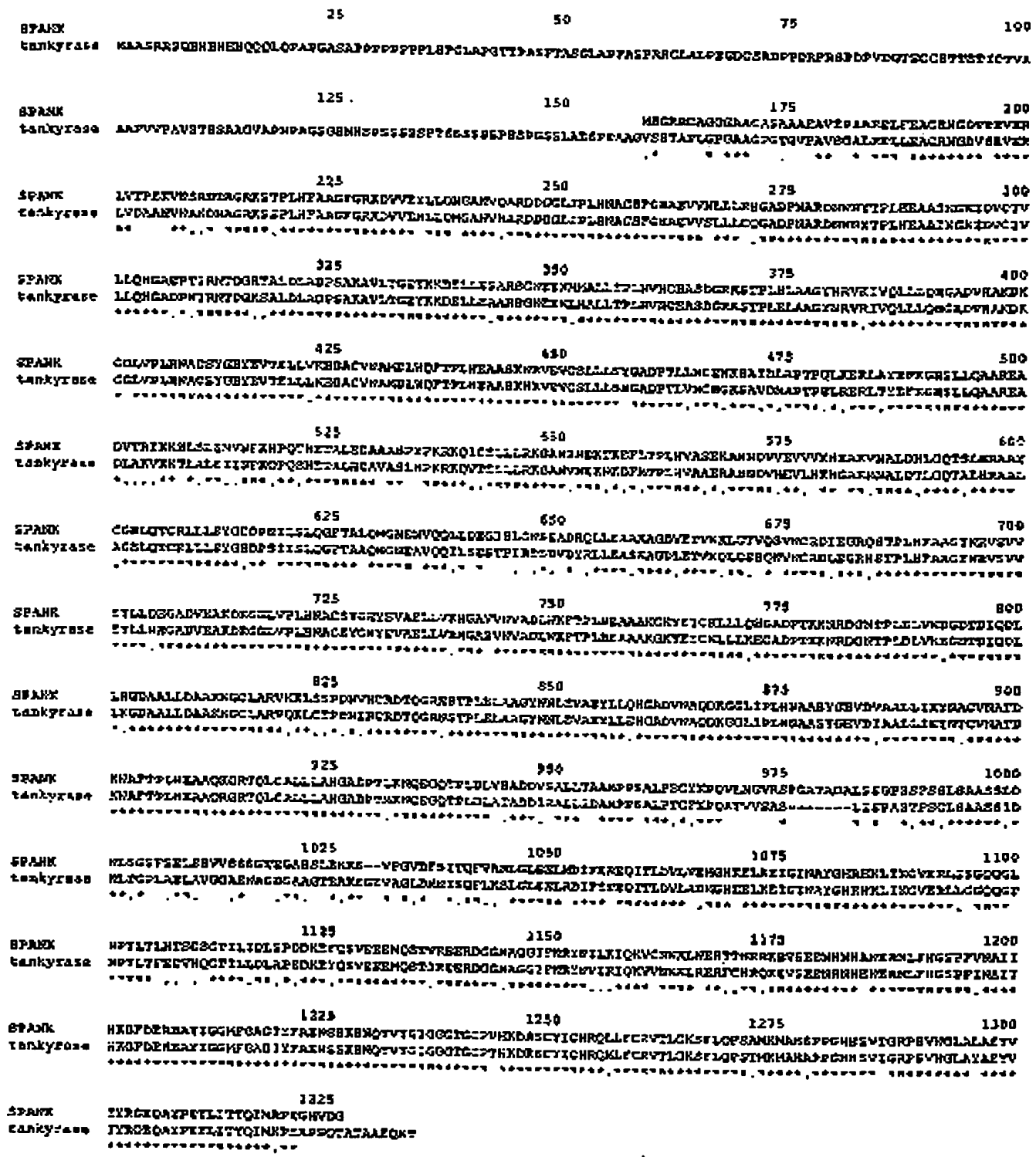

FIG 8